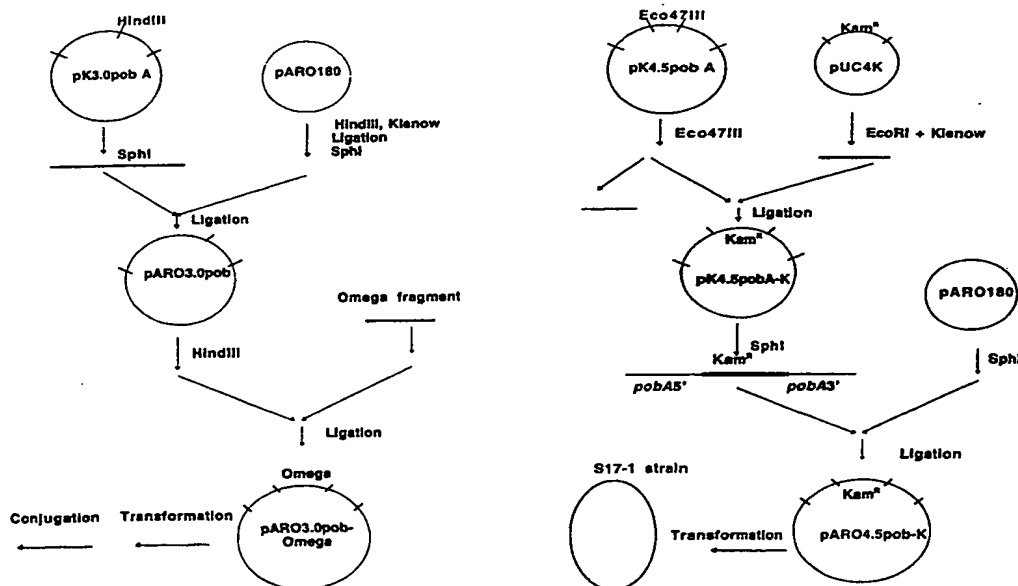




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(54) Title: METHOD FOR THE PRODUCTION OF PARA-HYDROXYBENZOATE IN *PSEUDOMONAS MENDOCINA*

(57) Abstract

A transformed mutant, *P. mendocina*, is provided containing a DNA fragment that inactivates all *pobA* genes and encodes the enzyme hydroxybenzoate hydroxylase. Mutants deficient in hydroxybenzoate hydroxylase are useful for the production of *para*-hydroxybenzoate (PHBA).

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TITLEMETHOD FOR THE PRODUCTION OF *PARA*-HYDROXYBENZOATE
IN *PSEUDOMONAS MENDOCINA*FIELD OF INVENTION

5 The present invention relates to the fields of molecular biology and microbiology and the use of genetic techniques to modify existing enzymatic pathways for the production of desired compounds. More specifically, the present invention relates to a method for the production of *para*-hydroxybenzoate (PHBA) by a toluene-metabolizing *Pseudomonas mendocina* mutant lacking the
10 ability to express *para*-hydroxybenzoate hydroxylase (PHBH).

BACKGROUND

Para-hydroxybenzoate (PHBA) is the key monomer for Liquid Crystal Polymers (LCPs) which contain approximately 67% PHBA. Esters of PHBA also can be used as backbone modifiers in other condensation polymers, i.e.,
15 polyesters, and are also used to make parabens preservatives.

 Chemical synthesis of PHBA is known. For example, JP 05009154 teaches a chemical route using the Kolbe-Schmidt process from tar acid and CO₂ involving the extraction of tar acid from a tar naphthalene oil by an aqueous potassium hydroxide; adding phenol to the extracted tar acid potassium salt;
20 removing H₂O and reacting the resultant slurry with CO₂. Alternative methods of synthesis are also known; see, for example, US 5,399,178; US 4,740,614; and US 3,985,797.

 Chemical synthesis is problematic and costly due to the high energy needed for synthesis and extensive purification of product required. An alternate
25 low cost method with simplified purification would represent an advance in the art. Biological production offers one such low cost, simplified solution to this problem.

 Microbiological methods of PHBA synthesis are known. For example, JP 06078780 teaches PHBA preparation by culturing benzoic acid in the presence
30 of microorganisms (preferably *Aspergillus*) that oxidize benzoic acid to PHBA.

 An alternate method of biological production is suggested by bacteria that have an enzymatic pathway for the degradation of toluene and other organics where PHBA is produced as an intermediate. The first enzyme in the toluene degradation pathway is toluene monooxygenase (TMO) and the pathway is
35 referred to as the TMO pathway. Bacteria that possess the TMO pathway are primarily restricted to the genus *Pseudomonas* where *P. putida*, *P. fluorescens*, *P. aeruginosa* and *P. mendocina* are the most commonly utilized species. The TMO pathway has been highly characterized [Romine et al., Bioremediation of Chlorinated Polycyclic Aromatic Hydrocarbon Compounds (1994), 271-6.

Editor(s): Hinchee, Robert E. Publisher: Lewis, Boca Raton, FL] and a number of the genes encoding key enzymes have been cloned and sequenced, including the protocatechuate 3,4-dioxygenase genes [Frazee, *J. Bacteriol.*, (1993), 175(19), 6194-202], the *pcaR* regulatory gene from *Pseudomonas putida*, which is required
5 for the complete degradation of *p*-hydroxybenzoate [Romero-Steiner et al., *J. Bacteriol.* (1994), 176(18), 5771-9; Dimarco et al., *J. Bacteriol.* (1994), 176(14), 4277-84] and the *pobA* gene encoding the expression of *para*-hydroxybenzoate hydroxylase (PHBH), the principal enzyme for the conversion of PHBA to protocatechuate [Wong et al., *Microbiology* (Reading U.K.) (1994),
10 140(10), 2775-86; Entsch et al., *Gene* (1988), 71(2), 279-91].

Bacteria that possess the TMO pathway are useful for the degradation of toluene and trichloroethylene and are able to use these and other organics as sole carbon sources where they are transformed through PHBA to ring opening degradation products (US 5,017,495; US 5,079,166; US 4,910,143).

15 Recently, various strains of *Pseudomonas* possessing the TMO pathway have been used to produce muconic acid via manipulation of growth conditions (US 4,657,863; US 4,968,612). Additionally, strains of *Enterobacter* with the ability to convert *p*-cresol to PHBA have been isolated from soil (JP 05328981). Further, JP 05336980 and JP 05336979 disclose isolated strains of *Pseudomonas*
20 *putida* with the ability to produce PHBA from *p*-cresol.

Although the above cited methods are useful for the production of PHBA, these methods are limited by the high cost and toxicity of the aromatic substrate, *p*-cresol. Furthermore, the above methods use an isolated wildtype organism that converts part of the *p*-cresol to PHBA while the rest is further metabolized. The
25 utility of these methods are therefore limited by low yields and an inability to control further degradation of the desired product.

Shuman et al., (*J. Biol. Chem.*, (1993), 268, 17057) have reported the identification of a gene encoding a putative isozyme of PHBH from
30 *P. fluorescens*. The comparison of the deduced amino acid sequence from this gene with the enzyme encoded by the more fully characterized *pobA* gene of *P. fluorescens* (van Berkel et al., *Eur. J. Biochem.*, (1992), 210 (2), 411-419) demonstrates 73% homology with the isozyme. Although the work of Shuman et al. (supra) suggest the presence of a second gene encoding an alternate PHBH, the authors were not able to isolate any expressed protein and concluded that the gene
35 encoding the isozyme is not expressed.

Therefore, the problem to be overcome is to develop a method of microbially mediated PHBA production from an inexpensive, aromatic substrate, where the microbial TMO pathway has been altered to prevent the degradation of PHBA.

SUMMARY OF THE INVENTION

The present invention provides a method for the production of PHBA comprising: (i) culturing a *pobA*(-) *Pseudomonas* strain in a medium containing an aromatic organic substrate, at least one suitable carbon source, and a nitrogen source, wherein the *pobA*(-) *Pseudomonas* strain comprises genes encoding the TMO toluene degradation pathway and wherein the *pobA*(-) *Pseudomonas* strain does not produce any detectable *para*-hydroxybenzoate hydroxylase, whereby PHBA accumulates; and (ii) recovering the PHBA.

The invention further provides genes encoding novel *para*-hydroxybenzoate hydroxylase enzymes and *pobA*(-) *Pseudomonas* strains deficient in hydroxybenzoate hydroxylase that are able to accumulate PHBA under suitable growth conditions and in the presence of a suitable aromatic substrate.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1(a) schematically represents plasmid vectors used for the disruption of *pobA-1* alone in the variant of *P. mendocina*.

Figure 1(b) schematically represents plasmid vectors used for the disruption of *pobA-1* and *pobA-2* in the variant of *P. mendocina*.

Figure 2 is schematically represents the plasmid constructs needed for inactivation of *pobA-1* and *pobA-2*.

Figure 3 graphically compares the accumulation of PHBA in the *pobA*(-) *P. mendocina* mutants.

Figure 4 schematically shows the TMO enzymatic pathway.

Figure 5 is a GC/MS analysis of PHBA production (PHBA peak indicated) from *P. mendocina* strains containing disrupted *pobA-1* and *pobA-2* genes.

BRIEF DESCRIPTION OF BIOLOGICAL DEPOSITS

AND SEQUENCE LISTING

The transformed *P. mendocina* KRC1651 containing the omega-disrupted *pobA-1* and *pobA-2* genes was deposited on November 26, 1996, with the ATCC under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and is designated as ATCC 55885.

The fast-growing *P. mendocina* KRC16 was deposited on November 26, 1996 with the ATCC under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and is designated as ATCC 55886.

"ATCC" refers to the American Type Culture Collection international depository located at 10801 University Boulevard, Manassas, VA 20110-2209, U.S.A. The designations refer to the accession number of the deposited material.

Applicants have provided 10 sequences in conformity with Rules for the Standard Representation of Nucleotide and Amino Acid Sequences in Patent Applications (Annexes I and II to the Decision of the President of the EPO, published in Supplement No. 2 to OJ EPO, 12/1992) and with

5 37 C.F.R. 1.821-1.825 and Appendices A and B (Requirements for Application Disclosures Containing Nucleotides and/or Amino Acid Sequences).

SEQ ID NO: 1 gives the nucleotide sequence of the *pobA-1* gene isolated from *P. mendocina* KR-1.

10 SEQ ID NO: 2 gives the nucleotide sequence of the *pobA-2* gene isolated from *P. mendocina* KR-1.

SEQ ID NO: 3 gives the amino acid sequence of the *pobA-1* gene isolated from *P. mendocina* KR-1.

SEQ ID NO: 4 gives the amino acid sequence of the *pobA-2* gene isolated from *P. mendocina* KR-1.

15 SEQ ID NO: 5 gives the nucleotide sequence of the *pobA* gene isolated from *P. fluorescens* (van Berkel et al., *supra*.)

SEQ ID NO: 6 gives the nucleotide sequence of the *pobB* gene encoding the PHBH isozyme isolated from *P. fluorescens* (Shuman et al., *supra*.)

20 SEQ ID NO: 7 gives the amino acid sequence of the *pobA* gene isolated from *P. fluorescens* (van Berkel et al., *supra*.)

SEQ ID NO: 8 gives the amino acid sequence of the *pobB* gene encoding the PHBH isozyme isolated from *P. fluorescens* (Shuman et al., *supra*.)

25 SEQ ID NO:9 gives the nucleotide sequence of the 5' primer used to generate the *pobA* hybridization probe for the isolation of the *pobA-1* and *pobA-2* genes.

SEQ ID NO:10 gives the nucleotide sequence of the 3' primer used to generate the *pobA* hybridization probe for the isolation of the *pobA-1* and *pobA-2* genes.

DETAILED DESCRIPTION OF THE INVENTION

30 Applicants have developed a fast-growing *P. mendocina* mutant which lacks the PHBH enzyme and is unable to convert PHBA to PCA. The mutant metabolizes toluene and *p*-cresol resulting in the accumulation of PHBA in the growth medium. Applicants have made the unexpected finding that *P. mendocina* possesses two, highly homologous *pobA* genes, both of which

35 encode enzymes able to convert PHBA to PCA.

Applicants have also provided a method for the biological production of PHBA from genetically altered *Pseudomonas*. The method provides PHBA without the high energy cost of synthetic production and without producing toxic

waste streams. PHBA is a valuable monomer for the synthesis of liquid crystalline polymers.

The following definitions are to be used to interpret the terms contained in the claims and specification.

5 The terms "plasmid" and "vector" refer to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA molecules. Such elements may be autonomously replicating sequences, genome-integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded
10 DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell. "Transformation vector" refers to a specific vector containing a foreign gene and
15 having elements in addition to the foreign gene that facilitate transformation of a particular host cell.

A "fragment" constitutes a fraction of the DNA sequence of the particular region.

20 The term "transformation" refers to the acquisition of new genes in a cell after the incorporation of nucleic acid.

 The term "expression" refers to the transcription and translation to gene product from a gene coding for the sequence of the gene product. In the expression, a DNA chain coding for the sequence of gene product is first transcribed to a complimentary RNA which is often a messenger RNA and, then,
25 the transcribed messenger RNA is translated into the above-mentioned gene product if the gene product is a protein.

 The terms "restriction endonuclease" and "restriction enzyme" refer to an enzyme which binds and cuts within a specific nucleotide sequence within double-stranded DNA.

30 "Polymerase Chain Reaction" and "PCR" refer to a method that results in the linear or logarithmic amplification of nucleic acid molecules. PCR generally requires a replication composition consisting of, for example, nucleotide triphosphates, two primers with appropriate sequences, DNA or RNA polymerase and proteins. These reagents and details describing procedures for their use in
35 amplifying nucleic acids are provided in U.S. 4,683,202 (1987, Mullis et al.) and U.S. 4,683,195 (1986, Mullis et al.).

 "Gene" refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding) and following (3' non-coding) the coding region. "Native" gene refers to an isolated gene with its own

regulatory sequences as found in nature. "Chimeric gene" refers to a gene that comprises heterogeneous regulatory and coding sequences not found so assembled in nature. "Endogenous" gene refers to the native gene normally found in its natural location in the genome and is not isolated.

5 "TMO" is the abbreviation for toluene monooxygenase-1.

The terms "TMO degradative pathway" or "TMO enzymatic pathway" refer to the enzymes and genes encoding the enzymes found in some *Pseudomonas* bacteria that are responsible for the degradation of toluene and similar aromatic substrates. The TMO pathway is outlined in Figure 4 and
10 contains at least toluene-4-monooxygenase, *p*-cresol methylhydroxylase, *p*-hydroxybenzaldehyde dehydrogenase, and *p*-hydroxybenzoate hydroxylase.

"PHBH" is the abbreviation for *para*-hydroxybenzoate hydroxylase, the enzyme in the TMO pathway responsible for the conversion of PHBA to PCA.

"PHBA" is the abbreviation for *para*-hydroxybenzoate which is also
15 known as *para*-hydroxybenzoic acid or 4-hydroxybenzoic acid.

"PCA" is the abbreviation for protocatechuic acid.

"EDTA" is the abbreviation for ethylenediaminetetraacetic acid.

The term "*pobA*" refers to a gene encoding the *para*-hydroxybenzoate hydroxylase enzyme.

20 The term "*pobB*" refers to the gene encoding the putative isozyme of PHBH from *P. fluorescens*.

"*pobA-1*" refers to a first gene encoding *para*-hydroxybenzoate hydroxylase and having the sequence as given by SEQ ID NO:1 and is contained within a 3.0 kb fragment of *P. mendocina* genomic DNA isolated by hybridization
25 using a *pobA* hybridization probe. "*pobA-2*" refers to a second gene encoding *para*-hydroxybenzoate hydroxylase and having the sequence as given by SEQ ID NO:2 and is contained within a 4.5 kb fragment of *P. mendocina* genomic DNA isolated by hybridization using a *pobA* hybridization probe.

30 "*pobR*" refers to a transcriptional activator gene required for the expression of *pobA* genes in *Pseudomonas* sp.

The term "*pobA* hybridization probe" refers to a 1.2 kb PCR amplification product produced by amplification of *P. mendocina* genomic DNA with primers based on the nucleotide sequence of the *P. fluorescens pobA* gene [SEQ ID NO: 5] as described by van Berkel et al., *Eur. J. Biochem.* (1992), 210 (2), 411-419.

35 The term "*pobA*(-) *Pseudomonas*" refers to a genetically manipulated *Pseudomonas* having mutations or disruptions in all *pobA* genes such that the genetically manipulated *Pseudomonas* is unable to produce active *para*-hydroxybenzoate hydroxylase.

The term "aromatic organic substrate" refers to an aromatic compound that is degraded by the TMO enzymatic pathway. Typical examples of suitable aromatic substrates are toluene and *p*-cresol.

5 The term "carbon source" refers to a substrate suitable for bacterial cell growth that is distinct from the aromatic substrate. Suitable carbon substrates include but are not limited to glucose and succinate.

10 The term "TMO *P. mendocina* variant" refers to a genetic variant of a wildtype *P. mendocina* capable of metabolizing toluene and demonstrating an increase in cell density of at least 6 fold greater than the wildtype under growth conditions of approximately 160 ppm toluene as a sole carbon source. Variants are typically produced by standard mutagenic methods and screened for higher growth rate and the ability to metabolize toluene and other aromatic substrates.

15 The term "suicide vector" refers to a vector generally containing a foreign DNA fragment to be expressed in a suitable host cell, coupled with a genetic element that will be lethal to the host cell unless the cell is able to express the foreign DNA. "Suicide vector" is also understood to mean a non-replicating vector capable of transfecting a host cell and facilitating the incorporation of foreign DNA into the genome of the host cell. Such a vector does not replicate and is thus destroyed after incorporation of the heterologous DNA. Examples of
20 common suicide vectors and their construction may be found in (Sambrook et al., Molecular Cloning: A Laboratory Manual - volumes 1,2,3 (Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1989)).

The term "*Kam*" refers to a gene encoding Kanamycin resistance.

The term "*Amp*" refers to a gene encoding Ampicillin resistance.

25 The term "*Strep*" refers to a gene encoding Streptomycin resistance.

Applicants' method involves mutagenesis of a toluene-metabolizing *P. mendocina* strain such that the mutant strain demonstrates a cell density of at least 6 fold greater than the wildtype under growth conditions of approximately 160 ppm toluene. The fast-growing TMO variant was then genetically altered so
30 that all genes encoding *para*-hydroxybenzoate hydroxylase (PHBH) were disrupted. *P. mendocina* strains containing the disrupted genes (*pobA-1*, *pobA-2*), referred to as *pobA*(-) mutants, were then grown in the presence of toluene or *p*-cresol and PHBA was accumulated in the medium.

35 Creation of *pobA*(-) mutants proceeded by (i) the isolation of a *pobA* hybridization probe; (ii) isolation of *pobA-1* from a TMO variant using the probe; (iii) disruption of *pobA-1* using an omega element and subsequent cloning and expression into a suicide vector which confirmed the existence of an alternate pathway for PHBA catabolism; (iv) isolation of *pobA-2*; (v) disruption of both *pobA-1* and *pobA-2*; and (vi) cloning into a suicide vector for the creation of a

pobA(-) mutant TMO variant that accumulates PHBA in the presence of toluene or *p*-cresol and an alternate carbon source.

One of the most significant aspects in the development of the present method is the discovery that the instant strains contained two expressed *pobA* genes, both of which needed to be inactivated before PHBA accumulation could be effected. Another significant aspect of the invention is the creation of a toluene-metabolizing *P. mendocina* strain that has a significantly higher rate of growth and metabolism than many toluene-metabolizing strains known in the art. The fast-growing TMO variant provides a host useful for the commercial production of PHBA and that permits rapid screening of *pobA*(-) transformants.

PRODUCTION CONDITIONS

TMO-Containing Bacterial Strains

Bacterial cells preferred in the present invention are those that possess the TMO pathway. Such strains are generally restricted to the genus *Pseudomonas* and include but are not limited to *P. putida*, *P. fluorescens*, *P. aeruginosa* and *P. mendocina*.

Strains of *Pseudomonas* containing the TMO pathway are known to oxidize toluene to form intermediates of the tricarboxylic acid cycle. PHBA as well as other intermediates, such as *p*-cresol, *p*-hydroxybenzyl alcohol and *p*-hydroxybenzaldehyde, are formed in the upper pathway, which metabolizes toluene to the ring cleavage substrate (Figure 4). In wildtype *Pseudomonas* strains, PHBA is immediately converted to protocatechuate (PCA) as it is formed. The biochemistry of the enzymes involved in the upper pathway have been described for several *Pseudomonas* strains (Romine et al., *supra*).

Batch and Continuous Fermentations

The present process uses a batch method of fermentation. A classical batch fermentation is a closed system where the composition of the medium is set at the beginning of the fermentation and not subjected to artificial alterations during the fermentation. Thus, at the beginning of the fermentation the medium is inoculated with the desired organism or organisms and fermentation is permitted to occur adding nothing to the system. Typically, however, a batch fermentation is "batch" with respect to the addition of carbon source and attempts are often made at controlling factors such as pH and oxygen concentration. In batch systems the metabolite and biomass compositions of the system change constantly up to the time the fermentation is stopped. Within batch cultures, cells moderate through a static lag phase to a high growth log phase and finally to a stationary phase where growth rate is diminished or halted. If untreated, cells in the stationary phase will eventually die.

A variation on the standard batch system is the Fed-Batch system-

Fed-Batch fermentation processes are also suitable in the present invention and comprise a typical batch system with the exception that the substrate is added in increments as the fermentation progresses. Fed-Batch systems are useful when catabolite repression is apt to inhibit the metabolism of the cells and where it is desirable to have limited amounts of substrate in the medium. An advantage of the Fed-Batch system is that it is more amenable to the use of toxic or immiscible aromatic substrates such as toluene. Using a Fed-Batch system it is possible to maintain a steady concentration of substrate at non-toxic levels while accommodating maximum bioconversion of the substrate to product.

The production of PHBA from aromatic compounds such as toluene will be limited by the amount of the aromatic substrate and carbon sources added. In simple Batch fermentation, production will be limited by the amount of toluene initially added. Since toluene is toxic and has limited solubility in water, its low initial concentration will govern the amount of PHBA produced. The ability to run the process at such a low toluene (i.e., 30-60 ppm) allows operation below a lower explosive limit which for toluene is 120 ppm. This is a clear safety advantage to the process. Fed-Batch techniques where the carbon source and toluene are added at rates which are similar to the utilization of these compounds will keep the toluene concentration in the medium low and can significantly increase the amount of PHBA produced.

Batch and Fed-Batch fermentations are common and well known in the art and examples may be found in, for example Thomas D. Brock in *Biotechnology: A Textbook of Industrial Microbiology*, Second Edition (1989) Sinauer Associates, Inc., Sunderland, MA., or Deshpande, Mukund V. *Appl. Biochem. Biotechnol.* 36, 227, (1992).

Although the present invention is performed in batch mode, it is contemplated that the method would be adaptable to continuous fermentation methods. Continuous fermentation is an open system where a defined fermentation medium is added continuously to a bioreactor and an equal amount of conditioned medium is removed simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density where cells are primarily in log phase growth.

Continuous fermentation allows for the modulation of one factor or any number of factors that affect cell growth or end product concentration. For example, one method will maintain a limiting nutrient such as the carbon source or nitrogen source at low concentration and allow all other parameters to be in excess. In other systems a number of factors affecting growth can be altered continuously while the cell concentration, measured by medium turbidity, is kept

constant. Continuous systems strive to maintain steady state growth conditions and thus the cell loss due to medium being drawn off must be balanced against the cell growth rate in the fermentation. Methods of modulating nutrients and growth factors for continuous fermentation processes as well as techniques for

5 maximizing the rate of product formation are well known in the art of industrial microbiology and a variety of methods are detailed by Brock, *supra*.

It is contemplated that the present invention may be practiced using either batch, Fed-Batch or continuous processes and that any known mode of fermentation would be suitable. Additionally, it is contemplated that cells may be
10 immobilized on a substrate as whole cell catalysts and subjected to fermentation conditions for PHBA production.

Fermentation Studies and Culture Conditions

The present invention provides a process for the production of PHBA in a culture medium by *P. mendocina* comprising culturing a genetically engineered
15 *P. mendocina* host having no *p*-hydroxybenzoate hydroxylase where all *pobA* genes are disrupted. The medium may optionally contain a nitrogen source, minor metals, a carbon source and an aromatic substrate. The culture medium can contain any inorganic or organic source of nitrogen normally used in processes for culturing microorganisms. Nitrogen sources may be either inorganic (ammonium salts, e.g., ammonium sulfate) or organic nitrogen (e.g., yeast extract).
20

Growth Conditions

Typically, cells are cultured in flasks and fermenters and the fermentation modes used were batch, Fed-Batch and continuous. Both "rich" and "lean" media were used as described in the GENERAL METHODS. Culture conditions were
25 modulated according to the method of growth and optimized for the production of PHBA. The pH of the cultures should be maintained within a range of about from 6.3 to 7.9 wherein a range from about 7.2 to 7.7 is most preferred. Methods for the optimization of cell growth and metabolism are well known in the art, for example as described in Manual of Methods for General Bacteriology (Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A.
30 Wood, Noel R. Krieg and G. Briggs Phillips, eds), American Society for Microbiology, Washington, DC. (1994).

Carbon Source

A variety of carbon sources are suitable in the present invention and
35 include but are not limited to materials such as succinate, lactate, acetate, ethanol, monosaccharides such as glucose and fructose, oligosaccharides such as lactose or sucrose, polysaccharides such as starch or cellulose or mixtures thereof and unpurified mixtures from renewable feedstocks such as cheese whey permeate, cornsteep liquor, sugar beet molasses, and barley malt. The choice of the carbon

substrate will be determined by the needs of the desired production cell. For the purposes of the present invention, glucose is preferred.

In *pobA(-)* *P. mendocina* cultures, a carbon source is necessary as the TMO pathway is completely blocked, and the strain is not able to utilize the aromatic substrate as a carbon source for the production of energy. In practice, it is necessary to balance the concentration of the carbon substrate against the concentration of the aromatic substrate in order to control the amount of oxidation of the aromatic substrate at the beginning of the TMO pathway.

Aromatic substrates

A variety of aromatic substrates may be used in the present invention, including but not limited to toluene, *p*-cresol, *p*-hydroxybenzyl alcohol, *p*-hydroxybenzaldehyde, benzoate and any aromatic compounds where the chemical structure is similar to toluene and the intermediates of the TMO pathway.

The production of PHBA from aromatic substrates such as toluene and *p*-cresol will be limited by the concentration of the substrate and the carbon source in the medium. Preferred concentrations of toluene are from about 30 ppm to about 500 ppm where a range of about 30 ppm to about 60 ppm is most preferred and a range of about 30 ppm to 60 ppm is most preferred. Preferred concentrations of *p*-cresol are from about 1 mM to about 5 mM.

CLONING AND SEQUENCING OF *pobA-1* GENE

Development of fast-growing TMO *P. mendocina* variant

The present invention provides a fast-growing *Pseudomonas* strain that exhibits a cell density of at least 6 fold greater than the wildtype under growth conditions of 0.2% toluene. The fast-growing strain is referred to as a TMO variant. Preferred *Pseudomonas* strains will be those that are able to metabolize toluene and possess the key enzymes in the TMO enzymatic pathway (Figure 4). Strains known to possess this pathway and useful in the present invention include but are not limited to *P. putida*, *P. fluorescens*, *P. aeruginosa* and *P. mendocina*. Most preferred are the *P. mendocina* strains KRC16 (ATCC 55886) and KRC50.

The TMO variant was developed by U.V. mutagenesis of a *P. menodcina* strain (KR-1) (Amgen, U.S. 5,171,684) known to be able to use toluene as a sole carbon source. After mutagenesis, cells were plated on a standard medium containing toluene and colonies were picked on the basis of size and speed of development. In this fashion KRC50 was isolated. A second mutation of KRC50 produced KRC16 (ATCC 55886) which exhibited a cell density of at least 6 fold higher than the parent KR-1 under growth conditions of approximately 160 ppm toluene. KRC16 was then used for further genetic studies.

Methods of mutagenesis useful in the invention are not limited to the use of U.V. light protocols but encompass a variety of agents such as chemical agents including HNO₂ and NH₂OH, as well as compounds that affect replicating DNA such as acridine dyes, notable for causing frameshift mutations. Specific methods for creating mutants using radiation or chemical agents are well documented in the art. See, for example, Brock, *supra*.

Isolation of *pobA-1* with a *pobA* hybridization probe

Identification of the *pobA-1* gene in the TMO variant KRC16 (ATCC 55886) proceeded by first amplifying genomic DNA from KRC16 with primers designed from the known base sequence of *pobA* isolated from *P. fluorescens* (van Berkel et al., *supra*). PCR amplification (U.S. 4,683,202 (1987, Mullis et al.) and U.S. 4,683,195 (1986, Mullis et al.) produced a 1.2 kb fragment that was used to probe KRC16 genomic DNA. Analysis of the genomic DNA by Southern blot (Southern, *J. Mol. Biol.* 98, 503, (1975)) revealed two bands by gel electrophoresis. One band, migrated at about 3.0 kb and the other migrated at 4.5 kb. The 3.0 kb band was excised and used for cloning and sequencing. Sequence analysis revealed the base sequence of *pobA-1* as given in SEQ ID NO:1. Comparison of the deduced amino acid sequence from the base sequence of *pobA-1* [SEQ ID NO:3] with the known peptide encoded by the *pobA* gene of *P. fluorescens* [SEQ ID NO:7] (van Berkle et al., *supra*) show 68% similarity suggesting *pobA-1* encodes *P. mendocina* PHBH.

Cloning *pobA-1* gene and confirmation of expression

The 3.0 kb fragment was re-isolated from agarose gel and cloned into an appropriate vector for transformation into *E. coli* in order to confirm *pobA-1* expression of active PHBH. Vectors suitable for the transformation of *E. coli* are numerous and well known in the art. Suitable vectors can be derived, for example, from a bacterium, a virus (such as bacteriophage T7 or a M-13 derived phage), a cosmid, a yeast or a plant. Protocols for obtaining and using such vectors are known to those in the art. (Sambrook et al. *Molecular Cloning: A Laboratory Manual* - volumes 1,2,3 (Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1989)).

PobA-1 was cloned into a *sphI* site of a pUC18 vector (Parke, D., *Gene* (1990), 93(1), 135-7), creating pUC3.0K which was used to transform the *E. coli* strain DH5 α . Transformants were screened for inserts containing the 3.0 kb fragment. Positive transformants were then grown in the presence of PHBA and toluidine to test for the expression of active PHBH by *pobA-1*. Conversion of PHBA to PCA was indicated by a colored reaction product formed by the reaction of toluidine and PCA. This method of assay is common and well known in the art; see, for example, "Application of *p*-Toluidine in Chromogenic Detection of

Catechol and Protocatechuate, Diphenolic Intermediates in Catabolism of Aromatic Compounds" (Parke, *Applied and Environmental Microbiology*, Aug. 1992, P.2694-2697).

DISRUPTION OF *pobA-1*

5 Cloning of the 3.0 kb *pobA-1* gene fragment into suicide vector

In order to clone the *pobA-1* gene into a suicide vector amenable to manipulation for gene disruption, the 3.0 kb fragment was re-isolated from pUC3.0k and cloned into the *sphI* site of the suicide vector, *pARO180* (Park, D., *supra*) to form the vector *pARO3.0pobA*. (Figure 1(a))

10 Construction of suicide vectors are common and well known in the art and have been developed to transfect a variety of microorganisms to facilitate genetic manipulations. For example, suicide vectors capable of transfecting *Salmonella* (Galen et al., 96th General Meeting of the American Society for Microbiology, New Orleans, Louisiana, USA, May 19-23, 1996. Abstracts of the General
15 Meeting of the American Society for Microbiology 96 (0), (1996) 529); *Streptococcus* (Brooker et al., *Letters in Applied Microbiology* 21 (5) (1995) 292-297); *Rhizobium* (Safronova et al., *Genetika* 30 (6) (1994) 763-768); *Rhodobacter* (Penfold et al., *Gene* (Amst) 118 (1) (1992) 145-146) and other
20 gram-negative bacteria (Skrzypek et al., *Plasmid* 29 (2) (1993) 160-163), have all been constructed. Suicide vectors will be constructed so as to accommodate the specific host to be transfected and to accomplish the desired purpose. Generally suicide vectors useful in the present invention are non-replicating and capable of
25 transfecting a host cell with foreign DNA and facilitating incorporation of that DNA into the genome of the host cell. Such vectors are destroyed after transfecting the desired host with foreign DNA.

Within the context of the present invention, a mobilizing, narrow host range vector, which will replicate to high copy number in *E. coli* is preferred. Such vectors will be able to be efficiently transferred to other gram-negative bacteria from a mobilizing host strain such as S17-1 (Parke et al., *supra*). For the
30 purposes of the present invention a variety of suicide vectors will serve to carry the *pobA* gene for transfection into a suitable host where *pARO180* is preferred.

Disruption of *pobA-1* gene with Omega element

In order to prevent expression of the cloned *pobA-1* gene, a nucleic acid disrupting fragment known as an omega element was cloned into a *HindIII* site
35 within the coding region of the *pobA-1* gene on the vector *pARO3.0pobA*. The omega element carries a gene for Streptomycin resistance and is fully described in Prentki et al., *Gene* 29, 303, (1984). The vector containing the disrupted *pobA-1* was then used to transform a mobilizing *E. coli* strain for eventual transfer into the *P. mendocina* KRC16 (ATCC 55886).

The process of gene disruption analysis is common and well known in the art. Gene disruption involves inserting a non-coding DNA fragment or a DNA fragment of a selectable marker in the coding region of a target structural gene and transforming a host containing an endogenous target gene with that disrupted construct. Homologous recombination between the disrupted gene and the endogenous gene will result in inactivation of the endogenous gene in the host organism. Gene disruption is used in a variety of organisms such as *Mycobacterium* (Balasubramanian et al., *Journal of Bacteriology* 178 (1) (1996) 273-279); *Saccharomyces* (Daran et al., *European Journal of Biochemistry* 233 (2) (1995) 520-530); *Brevibacterium* (Su Y-C et al., Proceedings of the National Science Council Republic of China Part B, Life Sciences 19 (2) (1995) 113-122); as well as *E. coli* (Cherepanov et al., *Gene* (Amsterdam) 158 (1) (1995) 9-14).

To transfer the plasmid containing the disrupted *pobA-1* gene to KRC16 (ATCC 55886), the transformed mobilizing *E. coli* was incubated in a 1:4 ratio with KRC16 and plated on a standard medium. Positive transformants were selected on the basis of antibiotic resistance and the presence of the disrupted *pobA-1* gene was confirmed by re-isolation of the gene by restriction analysis and hybridization with the 1.2 kb *pobA* hybridization probe. This process is called transformation by conjugation. Alternatively, cells may be transformed by known procedures such as by protocols involving calcium-permeabilized cells, electroporation, or by transfection using a recombinant phage virus. (Sambrook et al., *supra*)

The disrupted *pobA-1* gene is an integrating DNA fragment which has a DNA sequence with homology to a target site in the genome of the host cell so that the chimerical DNA fragment of *pobA-1* gene and a drug-resistance gene can be integrated into the genome of the host *P. mendocina*.

ANALYSIS OF PHBA CONVERSION WITH *pobA-1* DISRUPTED STRAINS

Transformed KRC16 (ATCC 55886) carrying the disrupted *pobA-1* gene was grown in the presence of toluene or *p*-cresol under suitable conditions for the production of PHBA. Analysis of the culture supernatant by GC/Mass spectrometry indicated that only marginal PHBA accumulation occurred while there was significant production of PCA. These data suggested that the disruption of *pobA-1* did not block the TMO pathway at the PHBH step and that the *P. mendocina* KRC16 (ATCC 55886) mutant carrying the disrupted *pobA-1* gene was still capable of converting PHBA to PCA.

Since the initial analysis of KRC16 genomic DNA had indicated a 4.5 kb fragment resulting from hybridization with the *pobA* probe, in addition to the 3.0 kb fragment, it was decided to investigate whether this 4.5 kb fragment

represented an alternate *pobA* gene capable of mediating the conversion of PHBA to PCA in the presence of a disrupted *pobA-1* gene.

CONSTRUCTION OF A *P. MENDOCINA* KRC16 CONTAINING TWO DISRUPTED *pobA* GENES

5 Isolation, disruption and cloning of *pobA-2*

The 4.5 kb fragment was isolated from a gel following restriction of *P. mendocina* genomic DNA and probing with the *pobA* hybridization probe, essentially in the same fashion as the isolation of the 3.0 kb fragment. The isolated gene was referred to as *pobA-2*. Sequence analysis revealed the base
10 sequence of *pobA-2* as given in SEQ ID NO:2. Comparison of the deduced amino acid sequence from the base sequence of *pobA-2* [SEQ ID NO:4] with the known peptide encoded by the *pobB* gene for a putative PHBH isozyme [SEQ ID NO:8] of *P. fluorescens* (Shuman et al., *supra*) show 71.2% similarity suggesting *pobA-2* encodes *P. mendocina* PHBH.

15 Disruption of *pobA-2*

In order to create a disrupted *pobA-2* gene the isolated *pobA-2* gene was cloned into a pUC4K vector (Pharmacia Biotech, Piscataway, NJ, 08854, USA) and then the gene was disrupted by the insertion of a *Kam* resistance gene cassette into an Eco47III site in the *pobA-2* gene. The resulting plasmid was termed
20 pK4.5pobA-Kam (Figure 1(b)). Digestion of pK4.5pobA-Kam with *sphI* and subsequent ligation with the suicide vector pARO180 produced pAR04.5pob-Kam. pAR04.5pob-Kam was moved into a mobilizing *E. coli* strain and KRC16, containing the omega-disrupted *pobA-1* gene was transformed. A number of transformants were selected and found to contain the disrupted *pobA-2*
25 gene. Eight strains (KRC1615, KRC1616, KRC1651 (ATCC 55885), KRC1617, KRC1645, KRC1652, KRC1653, and KRC1657) were selected for further study. The presence of both disrupted genes was confirmed by re-isolation of the genes by restriction analysis and hybridization with the 1.2 kb *pobA* hybridization probe.

30 PHBA ACCUMULATION IN KRC1615, KRC1616, KRC1651 (ATCC 55885), KRC1617, KRC1645, KRC1652, KRC1653, AND KRC1657

Eight *P. mendocina* strains, (KRC1615, KRC1616, KRC1651 (ATCC 55885), KRC1617, KRC1645, KRC1652, KRC1653, and KRC1657), containing disrupted *pobA-1* and *pobA-2* genes, were grown in the presence of toluene or *p*-cresol and an alternate carbon source and the culture supernatant was
35 analyzed for accumulation of PHBA and PCA. Analysis by GC/MS indicated that no PCA was formed. KRC1651 (ATCC 55885) appeared to be the highest producer of PHBA. A comparison of the relative PHBA production of all strains is seen in Figure 3.

METHODS OF PHBA ASSAY

PHBA may be detected by a variety of means including Gas chromatography coupled with Mass spectrophotometry (GC/MS) or methods using capillary electrophoresis and HPLC. Capillary electrophoresis is preferred for rapid analysis where a large number of samples are involved, whereas GC/MS is noted for its sensitivity, accuracy and the ability to confirm the correct chemical structure of the compound being characterized.

Methods for determining the existence of PHBA and toluene derivatives by GC/MS are known in the art (see, for example, Adachi et al., *Meijo Daigaku Nogakubu Gakujutsu Hokoku* (1996) 32, 53-57; Luczak et al., *Fitoterapia* (1991) 62(5), 455-6; Uda et al., *Nippon Shokuhin Kogyo Gakkaishi* (1988) 35(5), 360-6). The present methods are based on those of the literature and are more fully described in the GENERAL METHODS.

Methods for determining PHBA by capillary electrophoresis are also known in the art (see, for example, Chen et al., *Fenxi Huaxue* (1995) 23(11), 1281-3; Wu et al., *J. Chromatogr., A* (1995) 716(1 + 2), 291-301). Capillary electrophoresis may be used in the present invention to detect PHBA as well as the disappearance of toluene and *p*-cresol from the culture supernatant of a *pobA*(-) *P. mendocina* strain.

Capillary electrophoresis uses a combination of electrophoretic migration and electroosmotic flow to separate molecules based on differences in charge, size, isoelectric focusing and hydrophobicity.

REGULATION OF THE *pobR* REPRESSOR

Although the foregoing describes only the genetic manipulation of the *pobA* gene in the *P. mendocina* TMO pathway, it is contemplated that other regulatory genes may also be manipulated to effect PHBA and other TMO intermediate metabolite accumulation. One such regulatory gene is the *pobR* gene.

pobR is a transcriptional activator required for the expression of *pobA* gene (DiMarco et al., *J. Bacteriol.* (1993) 175(14), 4499-506; DiMarco et al., *J. Bacteriol.* (1994) 176(14), 4277-84). Applicants have isolated and sequenced two *pobR* genes from *P. mendocina* KRC16, which are located upstream of *pobA*-1 and *pobA*-2. It is possible that each *pobA* gene in *P. mendocina* contains a region which may be involved in sequence-specific recognition by *pobR*, forming the core of the operator site as reported in *Acinetobacter calcoaceticus* (DiMarco, (1994), *supra*). In the presence of an inducer such as PHBA, association of *pobR* with its ligand fosters binding by RNA polymerase and leads to transcription of *pobA*. It is contemplated that an alternative approach to generating a PHBA-producing *P. mendocina* strain would involve the manipulation of the *pobR* genes

controlling each respective *pobA* gene. For example, *pobR* genes could be genetically altered or disrupted so as to prevent transcription of all *pobA* genes, resulting in inhibited PHBH expression and accumulation of PHBA in the altered cells.

5 The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit
10 and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

EXAMPLES

GENERAL METHODS

Procedures for the genetic manipulations of cellular genomes are well
15 known in the art. Techniques suitable for use in the following examples may be found in Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989).

Materials and methods suitable for the maintenance and growth of bacterial cultures are well known in the art. Techniques suitable for use in the
20 following examples may be found in Manual of Methods for General Bacteriology (Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, eds), American Society for Microbiology, Washington, DC. (1994) or Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates,
25 Inc., Sunderland, MA. All reagents and materials used for the growth and maintenance of bacterial cells were obtained from Aldrich Chemicals (Milwaukee, WI), DIFCO Laboratories (Detroit, MI), GIBCO/BRL (Gaithersburg, MD), or Sigma Chemical Company (St. Louis, MO) unless otherwise specified.

The meaning of abbreviations is as follows: "h" means hour(s), "min"
30 means minute(s), "sec" means second(s), "d" means day(s), "mL" means milliliters, "L" means liters.

Cell strains:

The *Pseudomonas* strains KRC16 (ATCC 55886) and KRC50 were used for transformation and production of PHBA.

35 Growth Conditions

Typically, studies were conducted in flasks and 1&10-L fermenters and the fermentation modes used were batch, Fed-Batch and continuous. The medium most commonly used was a basal medium containing 0.48 g/L of yeast extract ("lean medium") or the same medium but with 4 g/L yeast extract and 10 g/L

N-Z-amine E ("rich medium" Sheffield Products, Kraft Inc.), glucose was the carbon source and ammonia the inorganic nitrogen source.

Rich medium is available commercially from a variety of sources as from GIBCO/BRL (Gaithersburg, MD) for example. Other media amenable to the procedures of the present invention are common in the art and are fully described in Manual of Methods for General Bacteriology (Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, eds), American Society for Microbiology, Washington, DC. (1994).

It is preferred that the cells are cultured in a 2-phase process, where the cells are first grown to a suitable cell density in rich medium in the first stage and then transferred to a production medium containing the aromatic substrate for PHBA production in a second stage. Preferred cell densities for the first and second stage culture are from about 0.1 g/L to about 50 g/L.

Analysis of Various Toluene Metabolites by Gas Chromatography

Compounds were prepared for GC analysis as follows: 10 mg of each compound was dissolved in 500 μ L of ethylacetate and 500 μ L of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA). This mixture was heated for 30 min at approximately 80 °C to allow derivatization of the compounds. The reaction was then allowed to cool down to room temperature and the volume was adjusted to 1.0 mL as needed. A 1:10 dilution was performed with all compounds and 100 μ L of each solution was dispersed into a GC vial. Samples were analyzed by injecting 1.0 μ L (1000 ng) into the column. The GC conditions used for analysis were: Initial temp. 100 °C for 5 min; increased to 200 °C over 20 min at 5 °C/min. The GC column used was HP-5 cross linked 5% Phenylmethyl siloxane (30 M x 0.32 ID). Using these conditions, the following retention times (min) were obtained for various compounds tested: *p*-cresol, 4.27; 4-hydroxybenzaldehyde, 10.22; 4-hydroxybenzylalcohol, 14.05; 4-hydroxybenzoic acid (PHBA), 17.12; protocatechuic acid (PCA), 21.74.

EXAMPLE 1

CLONING AND SEQUENCING OF *pobA-1* GENE

Mutagenesis of *P. mendocina* KR1

A colony of *P. mendocina* KR-1 (Amgen, Thousand Oaks, CA) was inoculated into about 3 mL LB medium and the culture shaken at 30 °C overnight. Cells were diluted by 100 to 500 fold with LB medium and plated on agar plates of LB medium. Plates were incubated at 30 °C for between 5 h to 10 h. The colonies were treated with UV (30 wt, distance: 60 inch) for about 2 to 2.5 h. Surviving cells (about 5%) were then incubated at 30 °C overnight and then plated on agar plates having minimal medium containing nitrogen source, minerals, and

salts. 20 μ L toluene as a sole carbon source was added and the cultures were incubated at 30 °C for at least two days. The largest colonies were scored on the basis of size and collected. These colonies were considered fast-growing variants of toluene-metabolizing KR-1.

- 5 One such variant was isolated and termed KRC50. KRC50 was again subjected to mutagenesis by U.V. light and a further isolation of variants was conducted. The second set of variants produced KRC16 (ATCC 55886), which also metabolized toluene and has a growth rate higher than either KR-1 or KRC50.

10 Isolation of a *pobA* hybridization probe

The *pobA* gene from *P. mendocina* was obtained by PCR for use as a hybridization probe in the isolation of *pobA* from KRC16 and KR-1.

- The primers 5'-CARTTRCTNCAYAANGTNGGNAT-3' [SEQ ID NO:9] and 5'-CTAYTCNATYTCYTCRTANRG-3' [SEQ ID NO:10], based on the
15 published sequence of the *pobA* gene from *P. fluorescens* [SEQ ID NO:5] (See, van Berkel, W.J.H. et al., *supra*) were used in a PCR reaction consisting of 100 μ L reaction mixture which contained: 0.5 mM dNTPs, reaction buffer (final concentration of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, and 0.01% gelatin), 0.1 μ g of *P. mendocina* genomic DNA, 1 unit of Taq® DNA
20 polymerase.

- The DNA sample was denatured at 94 °C for 1 min, and annealed at 45 °C for 2 min. Polymerization was done at 72 °C for 3 min with an increased extension time of 5 sec per cycle. The polymerase chain reaction was accomplished by 30 cycles. The PCR DNA fragment was detected and analyzed
25 by regular 1% agarose gel electrophoresis with ethidium bromide. A 1.2 kb PCR DNA fragment containing the *pobA* gene was isolated and cloned into the vector of PCRscript (Pharmacia Biotech, Piscataway, NJ).

Preparation of genomic DNA from *P. mendocina* for isolation of *pobA-1*

- 50 mL of culture medium was used for the isolation of *P. mendocina*
30 genomic DNA. The cells of an overnight culture were spun down at 6000 rpm, 4 °C for 10 min. Supernatant was decanted and the pellets were resuspended with 5 mL TEG (25 mM Tris-HCl, 10 mM EDTA, 50 mM glucose, pH8). About 1.5 mL of RNase (100 ug/mL) was added into the mixture. The sample was kept at room temperature for 5 min, and then extracted with phenol twice. The two
35 phases were separated by a centrifugation at 6000 rpm for 10 min. The aqueous phase was extracted with phenol:chloroform twice. Two volumes of 100% ethanol was added to the aqueous phase to precipitate DNA. After 20 min the solution was centrifuged at 10,000 rpm, and the pellet was collected, dried, and resuspended in about 2 to 5 mL TE buffer (10 mM Tris-HCl, 1 mM EDTA,

pH 7.6). The DNA samples were then dialyzed against TE buffer at 4 °C overnight.

Identification of *pobA-1* gene from *P. mendocina* genomic DNA

10 µg of *P. mendocina* genomic DNA was used for Southern blot analysis using the *pobA* hybridization probe described above. DNA samples were first digested with EcoRI, HindIII and SphI and the digests were analyzed by 1% agarose gel electrophoresis. The DNA samples were then transferred to nitrocellulose membrane by denaturation and neutralization as described previously. The membranes were exposed to U.V. light for about 2 min to produce nucleic acid crosslinking. Prior to crosslinking the membrane had been prehybridized for about 1 to 2 h at 65 °C in a solution containing 5 X SSC, 0.1% (w/v) SDS, 0.5% (w/v) blocking reagent (DuPont, Wilmington, DE) and 5% (w/v) Dextran Sulfate.

For ease of identification the 1.2 kb DNA *pobA* probe was labeled with a fluorescein nucleotide in a 30 µL reaction mixture containing: a random primer, reaction buffer, fluorescein nucleotide mix and Klenow enzyme at 37 °C for 1 h. The labeled probe was then hybridized to the membrane-bound genomic DNA in the same buffer for 16 h at 65 °C.

After hybridization, the membrane was washed for 15 min in 2 X SSC, 0.1% SDS, followed by a second 15 min wash in 0.2 X SSC, 0.1% SDS at 65 °C. The membrane was then blocked for 1 h in buffer containing 0.5% blocking reagent and then incubated with antiluorescein HRP conjugate (1:1000) at room temperature for 1 h.

After the incubation the membranes were washed four times for 5 min with 0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, and incubated in a chemiluminescence reagent (Renaissance, nucleic acid chemiluminescent reagent, DuPont, Wilmington, DE) for 1 min at room temperature, and then exposed to the REFLECTION™ film (DuPont, Wilmington, DE). In this manner portions of the genomic DNA hybridizing to the *pobA* probe were isolated.

Cloning the *pobA-1* gene

A partial genomic DNA library of *P. mendocina* KR1 was prepared. Genomic DNA was restricted with SphI which produced fragments of about 3.0 kb. The fragments were recovered using the DNA preparation kit, GeneClean Bio101 (Vista, CA). The SphI fragments were ligated into a SphI digested pUC18 vector (Pharmacia Biotech, Piscataway, NJ) and the recombinant plasmids were used to transform Dh5-a *E. coli*. Analysis of the transformants by miniprep and mini agarose gel electrophoresis (Sambrook, *supra*) indicated that about 80% of plasmid DNA contained an insert of about 3.0 Kb in length. Plasmids containing the 3.0 kb insert were termed pUC3.0k.

Confirmation and Isolation of *pobA-1* with toluidine

The correct isolation of *pobA-1* was confirmed using a colorimetric assay essentially as described by Parke ("Application of *p*-Toluidine in Chromogenic Detection of Catechol and Protocatechuate, Diphenolic Intermediates in

- 5 Catabolism of Aromatic Compounds", *Applied and Environmental Microbiology*, Aug. 1992, 2694-2697).

Transformants containing the 3 kb insert were selected from the agar plates with ampicillin and were transferred onto the agar plates containing minimal medium, supplemented with 50 µg *p*-toluidine, 1 mM PHBA, 2.5 mM
10 proline and 1 µg/mL thiamine and iron. The plates were incubated at 37 °C overnight.

Activity of *pobA-1* and the production of protocatechuate was confirmed by the appearance of a color on the plates.

Sequencing of the *pobA-1* gene

- 15 The plasmid of pUC3.0k *pobA* was used for DNA sequencing. The DNA sequencing with synthetic primers was done at The Biopolymer Laboratory, Department of Microbiology and Immunology at the University of Maryland, School of Medicine according to standard methods. Accordingly the sequence of *pobA-1* was determined and is given as SEQ ID NO:1.

20

EXAMPLE 2

CLONING OF *pobA-1* INTO A SUICIDE VECTOR AND TRANSFORMATION OF *P. MENDOCINA*

Cloning of the 3.0 kb *pobA* gene fragment into suicide vector pARO180

- 25 The 3.0 kb *pobA* gene fragment was isolated from pUC3.0k by digesting the plasmid with SphI and further isolation by gel electrophoresis. The isolated 3.0 kb restriction fragment of *pobA* gene was then ligated with the SphI digested mobilizable suicide vector pARO180 (Parke, *Gene* (1990), 93(1), 135-7) to produce pARO3.0pobA. Plasmid pARO180pobAΩ contains an omega fragment inserted into the HindIII site of the first *pobA* gene on a 3.0 kb SphI
30 DNA fragment.

Prior to ligation the HindIII site adjacent to the SphI site on pARO180 was destroyed using a Klenow fragment (Figure 1(a)) pARO3.0pobA was then used to transform Dh5α *E. coli*.

Disruption of the *pobA-1* gene with Omega element

- 35 A 2.0 kb HindIII restriction fragment of omega element (Prentki et al., *Gene* 29, 303, (1984), was inserted into the HindIII site of pARO3.0pobA which is located within the coding region of the *pobA* gene. The resulting plasmid was designed as pARpobA omega.

Introduction of the disrupted *pobA* gene into *P. mendocina* strain KRC16

The mobilizing *E. coli* strain S17-1 (Parke et al., *supra*) was used to introduce the disrupted *pobA-1* gene containing the omega element into *P. mendocina* KR16 (Figure 2).

- 5 pARpobA omega containing the 3.0 kb omega-disrupted *pobA-1* was used to transform KRC16 using a standard transformation procedure as described below. A single colony of S17-1 strain having the plasmid of pobAomega was inoculated in 20 mL LB medium, and grown at 37 °C until log phase. Additionally untransformed *P. mendocina* strain KR16 was also inoculated in
- 10 20 mL LB medium and incubated at 30 °C until log phase. The cells of both strains were washed with LB medium two times and resuspended in LB medium. A mixture of donor and recipient cells [S17-1 cells and *P. mendocina* KR1] at a ratio of 1:4 was plated on the agar plates of LB medium. The plates were incubated at 30 °C for 8 h. The cells were collected and then plated on the agar
- 15 plates containing phosphate buffer, 1 mM succinate, 10 mM streptomycin. The colonies of *P. mendocina* strain which can grow on the selection plates were scored and collected. Positive transformants were designated KRC16::pobAΩ (Figure 2).

Identification of the disruption of *pobA-1*

- 20 Genomic DNA was isolated from wildtype and mutant KRC16::pobAΩ *P. mendocina* and restricted with SphI and then subjected to 1% agarose gel electrophoresis. After gel electrophoresis, the DNA samples were transferred to nylon membrane, and denatured and neutralized as described previously. The membranes were exposed to UV for about 2 min and the membrane was
- 25 prehybridized for about 1 to 2 h at 65 °C in a solution containing 5 X SSC, 0.1% (w/v) SDS, 0.5% (w/v) blocking reagent (DuPont, Wilmington, DE) and 5% (w/v) Dextran Sulfate.

- The 1.2 kb DNA fragment of *pobA-1* (Example 1) was labeled with fluorescein nucleotide in a 30 μL reaction mixture containing random primer,
- 30 reaction buffer, fluorescein nucleotide mix and Klenow enzyme at 37 °C for 1 h. Hybridization to labeled probe (16 h at 65 °C) was done by using the above-described solution.

- After hybridization, the membrane was given one 15-min wash in 2 X SSC, 0.1% SDS, and one 15-min wash in 0.2 X SSC, 0.1% SDS (65 °C). The
- 35 membrane was blocked for 1 h in buffer of 0.5% blocking reagent and then incubated with anti fluorescein HRP conjugate (1:1000) at room temperature for 1 h. After the membrane was washed for 5-min. with buffer of 0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, it was incubated in the chemiluminescence reagent for 1 min at room temperature, and then exposed to the REFLECTION™ film.

Analysis of the exposed film confirmed the presence of the disrupted *pobA-1*, seen as a shift in the mobility from the 3.0 kb fragment of the wildtype.

EXAMPLE 3

ANALYSIS OF PHBA PRODUCTION IN *pobA-1(-)* *P. MENDOCINA*

5 Example 3 demonstrates the conversion of toluene to PHBA by *P. mendocina* strains KRC16::*pobA*Ω, and KRC16.

Organisms were initially grown on LB agar plates containing (gr): tryptone, 10.0; yeast extract, 5.0; sodium chloride, 10.0; agar Noble, 15.0; double distilled H₂O, 1.0 liter (pH ~6.6). Plates were incubated for ~60 h at 30 °C and
10 were then stored at 4 °C. One liter flasks (x20) each 125 mL center-well flask contained 25 mL of the PHBA medium containing (gr): sodium phosphate, monobasic, 1.85; potassium phosphate, dibasic, 5.84; ammonium sulfate, 1.50; glucose, 1.0; yeast extract, 0.25; tryptone, 0.25; double distilled water, 1.0 liter; (pH 7.0-7.2) and 250 μL of trace minerals. The trace minerals solution contained
15 the following which were added to ~50 mL of double distilled H₂O containing 1.0 mL of concentrated HCl, (in mg): MgCl₂, 6H₂O, 2.50; CaCl₂, 2H₂O, 1.00; MnCl₂, 4H₂O, 400; Na₂MoO₄, 2H₂O, 750; FeSO₄, 7H₂O, 100; CuSO₄, 5H₂O, 100; ZnSO₄, 7H₂O, 100. The volume was adjusted to 100 mL. Trace mineral solution (1.0 mL) was added to 100 mL of the minimal medium after autoclaving.
20 Toluene (500 μL) was added to the center-well. Cells were grown for 24 h. Culture suspensions (5 mL) were then used to inoculate 200 mL of the fresh medium in one liter flasks. These were incubated with shaking for an additional 24 h. Culture supernatant (50 mL) of each strain was acidified to pH 2-3 and extracted (x3) with ethylacetate (50 mL), dried over magnesium sulfate and
25 evaporated to dryness under a stream of nitrogen. Samples were resuspended in ethylacetate (500 μL) and BSTFA (500 μL) was added. The vials were capped and heated to ~80 °C for 30 min to allow for complete derivatization. Samples were then transferred to GC vials and the volume was adjusted to 1.0 mL and were analyzed by GC as outlined above.

30 GC analysis showed the presence of PHBA in cultures of KRC16::*pobA*Ω cells grown on toluene. This sample was then submitted for GC/MS analysis for further confirmation of the presence of PHBA and PCA.

EXAMPLE 4

DETERMINATION OF THE PRESENCE OF AN ALTERNATE *pobA* GENE

35 Conversion of PHBA to Protocatechuate by *P. mendocina* Strains KR16::*pobA*Ω and KR1 Following Growth on *p*-Cresol

Since strain KR1::*pobA*Ω contains the interrupted *pobA* gene, this organism is expected to be incapable of converting PHBA to protocatechuate (PCA).

Organisms were grown on Laura Broth agar (LB) plates. Flasks (8 x 125 mL) each containing 25 mL of PHBA minimal medium were set up and *p*-Cresol (1 mM filter-sterilized). Flasks were inoculated with colonies from 1.5 plates per flask and were incubated at 30 °C on the shaker (200 RPM) for ~24 h. Cells obtained, (KR16*pobA*Ω, 6.26 g, and KR1, 6.37 g) were then stored at -80 °C until needed. The PEG buffer contained: 50 mM sodium phosphate buffer plus 10% glycerol, 1.0 mM DTT and 0.2 mM MgCl₂. Cells were then disrupted by passing twice through the French Pressure cell (3/8", 20,000 PSI). The cell debris was removed by centrifugation for 25 min at 38,000 x g at 4 °C. PMSF (phenylmethanesulfonyl fluoride) was added for a final concentration of 200 micro Molar after the cells were disrupted. The second pass through the French Pressure Cell was followed with a 3.0 mL rinse with PEG buffer. The protein concentration in the crude extract was determined using the Bicinchoninic acid (BCA) method as described by the manufacturer (Pierce, Rockford, IL).

15 The Effect of Buffers on the Rate of PHBA Hydroxylation

To study the effect of various buffers on the rate of PHBA hydroxylation, two separate buffers, Tris/HCl pH 8.0 and 50 mM sodium phosphate pH 6.8 buffers were used. The incubation mixtures for conversion of PHBA to PCA contained: NADPH, 6.0 μmoles; PHBA, 11.6 μmoles; FAD, 1.0 μmole and 2.0 mg protein of each extract (KR16::pobAΩ and KR1) and buffer to a total volume of 5 mL. After protein addition, the reaction mixtures were incubated with shaking at 30 °C for 5 min, stopped by the addition of 2 drops concentrated HCl, and extracted (x3) with diethyl ether (5 mL). The organic pool was dried over Na₂SO₄ and evaporated to dryness under a stream of nitrogen. The data is summarized below in Table 1.

TABLE 1

Organism/Buffer	PCT formed μg/mL	PCA formed nmole/mL
KR16::pobAΩ Na-phosphate	22	143
KR1 Na-phosphate	12.5	81
KR1::pobAΩ Tris-HCl	18.7	121
KR1 Tris-HCl	18.8	122

As can be seen by the data in Table 1, the PHBA hydroxylase is still active in KR16::pobAΩ because PCA is being formed from PHBA by this organism at levels comparable to the wild type (KR1) organism. No significant difference was observed in the amount of PCA formed with either phosphate or Tris buffer.

Conversion of PHBA to PCA by *P. mendocina* strains, KRC16::pobA Ω , KRC16, and KR1 when grown on *p*-Cresol

Since strain KRC16::pobA Ω , KRC16, and KR1 contain the interrupted *pobA* gene, these organisms are expected to be incapable of converting PHBA to PCA.

Using a single loop of colonies from the agar plates, flasks containing *p*-cresol (1 mM), were inoculated and allowed to grow with shaking for 24 h at 30 °C. These cultures were used to inoculate 200 mL of medium containing *p*-cresol (1.0 mM). Organisms were harvested after 24 h, washed, and the crude extract was obtained as described above.

Conversion of PHBA to PCA

Incubation mixtures contained: NADPH, 6.0 μ moles; PHBA, 11.6 μ moles; FAD, 1.2 μ moles; crude extract, 2.0 mg; Tris/HCl, pH 8.0 to final volume of 5.0 mL. Reaction mixtures were incubated for 5 min at 30 °C on shaker and were then stopped with 2 drops of concentrated HCl. The contents of the vials were extracted and prepared for GC analysis as explained above. Tests were performed in duplicates. Table 2 summarizes the data obtained.

TABLE 2

Organism	PCA formed μ g/mL	PCA formed μ moles/mL
KRC16::pobA Ω	19.5	127
KRC16	24.3	159
KR1	21.6	141

As can be seen by Table 2, PHBA hydroxylase enzyme is active in all three organisms. KRC16::pobA Ω , in which the *pobA* gene was interrupted, is still capable of converting PHBA to PCA. This data implies the presence and functioning of more than one PHBA hydroxylase encoding gene (*pobA*) in *P. mendocina* KR1.

Effect of EDTA on PCA Accumulation in Cultures

The ability of *P. mendocina* strains KRC16::pobA Ω , KRC16, and KR1 to convert PHBA to PCA when grown on *p*-cresol was verified and the effect of ethylenediaminetetraacetic acid (EDTA) on the accumulation of PCA was studied. EDTA was expected to prevent further degradation of PCA and thus allow for its accumulation. Organisms were grown on *p*-Cresol (1.0 mM) and the crude extract was obtained as described before.

The reaction mixtures, which were set up in duplicates, contained: NADPH, 6.0 μ moles; PHBA, 11.6 μ moles; FAD, 1.2 μ moles; EDTA, 3.0 μ moles;

protein, 2.0 mg; sodium phosphate buffer (50 mM) pH 6.8 to final volume of 5.0 mL.

Reaction mixtures were incubated for 5 min at 30 °C on the shaker and were terminated with 2 drops of concentrated HCl. Contents of the vials were
5 extracted and prepared for GC analysis as before. Table 3 summarizes data for analysis.

TABLE 3

Organism	PCA formed $\mu\text{g/mL}$	PCA formed $\mu\text{moles/mL}$
KRC16:: <i>pobA</i> Ω	9.9	64
KRC16:: <i>pobA</i> Ω (-EDTA)	9.3	61
KRC16	19.9	78
KR1	15.1	98

As can be seen in Table 3, PHBA hydroxylation is active in all organisms,
10 and addition of EDTA resulted in about 5% increase in the amount of PCA formed.

EXAMPLE 5

CLONING AND SEQUENCING OF *pobA-2*

Cloning of *pobA-2* gene

15 A partial genomic DNA library of *P. mendocina* KRC16, was constructed as follows. Genomic DNA was restricted with SphI and 4.5 kb restriction fragments were isolated and recovered from an agarose gel using a DNA preparation kit, (GeneClean Bio101, Vista, CA). Isolated DNA was then ligated
20 into pUC18 at the appropriate SphI restriction sites and used to transform *E. coli* Dh5 α .

Miniprep and mini agarose gel electrophoresis indicated that about 80% of the transformants contained an insert of about 4.5 Kb in length.

Identification and isolation of *pobA-2* by colony hybridization

25 The positive transformants were first grown on the LB agar plates overnight. The plates were chilled to 4 °C for one h. The colonies were then transferred onto the membrane discs. The membrane discs were treated with 0.5 N NaOH twice 2-min, and then washed twice for 2-min with 1 M Tris-HCl, pH 7.5. The DNA was fixed on the membrane by UV crosslinking. Membranes
30 were prehybridized for about 1 to 2 h at 65 °C in a solution containing 5 X SSC, 0.1% (w/v) SDS, 0.5% (w/v) blocking reagent (DuPont, Wilmington, DE) and 5% (w/v) Dextran Sulfate.

The 1.2 kb DNA fragment of *pobA* gene, constituting the *pobA* hybridization probe, was labeled and used to detect the presence of *pobA-2* in

essentially the same manner as it was used to confirm the presence of *pobA-1* as described in Example 1.

The plasmid DNA was isolated from the positive colony detected with colony hybridization. The size of the insert was determined by the SphI digestion and miniagarose gel electrophoresis.

Sequencing of the *pobA-2* gene

The plasmid of pUC4.5k *pobA* was used for DNA sequencing. The DNA sequencing with synthetic primers was done at The Biopolymer Laboratory, Department of Microbiology and Immunology at the University of Maryland, School of Medicine according to standard methods. Accordingly the sequence of *pobA-2* was determined and is given as SEQ ID NO:2.

EXAMPLE 6

CONSTRUCTION OF THE DISRUPTED *pobA-2* AND CLONING INTO A SUICIDE VECTOR

Disruption of *pobA-2* gene by deletion and insertion

The Kam resistance gene cassette was removed from the plasmid pUC4K (Pharmacia Biotech, Piscataway, NJ) by digesting with EcoRI (Figure 1(b)). Sticky ends were then repaired with Klenow. pK4.5pobA, containing the 4.5 kb fragment encoding pobA was then digested with Eco47III, which resulted in a Eco47III fragment being deleted from the coding region of *pobA-2* on the 4.5 kb SphI fragment (Figure 1(b)).

The Kam resistance gene cassette was then inserted into the Eco47III digested pK4.5pobA plasmid to form the plasmid of pK4.5pobA-Kam. The orientation of the Kam gene cassette in the pobA gene was determined by the restriction mapping of the resulted plasmid.

Cloning of the disrupted *pobA-2* gene into suicide vector

The disrupted *pobA-2* gene with Kam gene cassette was digested with SphI and the resulting DNA fragment was ligated with the SphI digested pARO180 plasmid to produce pAR04.5pob-K, which was then used to transform S17-1 *E. coli* (Figure 1(b)). Positive colonies were selected on the agar plates on the basis of Kam resistance.

Introduction of the disrupted *pobA-2* gene into the mutant of *P. mendocina* KRC16::*pobA*Ω

A single colony of S17-1 strain having the plasmid of pARP4.5pobA-Kam (Figure 1(b)) was inoculated in 20 mL LB medium, and grown at 37 °C to log phase. Another colony of KRC16::*pobA*Ω was inoculated in 20 mL LB medium and incubated at 30 °C and grown to log phase.

The cells of both cultures were washed twice with LB medium and resuspended in LB medium. S17-1 cells and *P. mendocina* KRC16::*pobA*Ω were

mixed at a ratio of 1:4 and were plated on agar plates of LB medium. The plates were incubated at 30 °C for 8 h. The cells were collected and then plated on the agar plates containing phosphate buffer, 1 mM succinate, 10 mM streptomycin and 25 µg/mL Kanamycin, and Kan-resistant colonies were selected.

5 Selection *P. mendocina* having a disruption in both *pobA-1* and *pobA-2*

A set of agar plates containing various substrates were used to select the mutants. The substrates were toluene, 20 µL for 30 mL medium, 1 mM *p*-cresol, 1 mM PHBA or 1 mM PCA respectively. The colonies were plated on these four plates containing different substrates as sole carbon source. Only the colonies of the mutants which cannot grow in the presence of PHBA and either toluene or *p*-cresol were scored while all of the colonies were grown with PCA. A similar experiment for further confirmation of the mutants was also conducted with liquid minimal medium containing the various substrates such as toluene (200 µL for 20 mL medium), 1 mM *p*-cresol, 1 mM PHBA or 1 mM PCA. The cell density was monitored with a spectrophotometer. The mutants which did not utilize toluene and PHBA in the liquid minimal medium were identified as the *P. mendocina* strain having a disruption of both *pobA-1* and *pobA-2* genes.

10 Identification of the disruption of *pobA-1* and *pobA-2* genes the *pobA(-)*

15 *P. mendocina* strain

20 Genomic DNA was isolated from the mutant strain of KRC1651 (ATCC 55885). DNA from both wild type and mutant cells were digested with SphI and then subjected to 1% agarose gel electrophoresis. After gel electrophoresis, the DNA samples were transferred to nylon membrane, and denatured and neutralized as described previously. The membranes were exposed to the UV for about 2 min for UV crosslinking of nucleic acids. Membranes were prehybridized for about 1 to 2 h at 65 °C in a solution containing 5 X SSC, 0.1% (w/v) SDS, 0.5% (w/v) blocking reagent (DuPont, Wilmington, DE) and 5% (w/v) Dextran Sulfate. The 1.2 kb DNA fragment of *pobA* gene was labeled with fluorescein nucleotide in a 30 µl reaction mixture containing random primer, reaction buffer, fluorescein nucleotide mix and Klenow enzyme at 37 °C for 1 h. Hybridization to labeled probe (16 h at 65 °C) was done by using the above-described solution. After hybridization, membrane was given one 15-min wash in 2 X SSC, 0.1% SDS, and one 15-min wash in 0.2 X SSC, 0.1% SDS (65 °C). The membrane was blocked for 1 h in buffer of 0.5% blocking reagent and then incubated with anti-fluorescein HRP conjugate (1:1000) at room temperature for 1 h. After the membrane was washed four 5-min with buffer of 0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, it was incubated in the chemiluminescence reagent for 1 min at room temperature, and then exposed to the REFLECTION™ film.

EXAMPLE 7PRODUCTION OF PHBA WITH *pobA* (-) *P. MENDOCINA*Conversion of Toluene to PHBA by the *pobA*(-) mutants *P. mendocina* Strains KRC1615, KRC1616 and KRC1651

5 Example 7 was designed to determine the ability of *P. mendocina* KRC1615, KRC1616 and KRC1651 *pobA*(-) mutants to convert toluene to pHBA when grown on succinate. The PHBA minimal medium with the following changes was used throughout the experiment: toluene, glucose, and tryptone were omitted and sodium succinate (at 1% final conc.) and yeast extract (at 0.01% final
10 conc.) were added (pH 7.1). Cells were harvested and washed after 21 h of growth. Center-well flasks were set up with the following additions: medium, 22.0 mL; trace mineral, 250 μ L; toluene, 750 μ L (center-well only); organisms, 300 mg (wet weight). Flasks were incubated with shaking at 30 °C. Samples were prepared for GC analysis as before. GC/MS analysis was performed and
15 results are attached as Figure 5.

As can be seen in Figure 5, no PCA was detected in any samples. PHBA was detected in some incubations after 50 h (strains KRC1615, KRC1616 and KRC1651) and after 95 h (KRC1616) as indicated below in Table 4:

20

TABLE 4

Strain	Time (h)	μ g PHBA formed
KRC1615	50	39.5
KRC1651	50	770
KRC1651*	50	4500
KRC1616	95	39.8
KRC1616*	95	58.8

* Indicates duplicates

As demonstrated by the data in Table 4 strain KRC1651 was most active in PHBA production.

25 These data indicate that strain KRC1651 is capable of converting toluene to PHBA and accumulating it. This implies that the pathway to form PCA from PHBA has been blocked in this organism. Further experiments on PHBA accumulation were therefore planned using *P. mendocina* KRC1651.

30 Effect of Glucose, Succinate and $MgCl_2$ on Growth and PHBA Production by the *pobA*(-) mutant KRC1651

The center-well flasks containing 200 mL of Luria Broth (LB) medium: Tryptone (1%); yeast extract (0.5%); sodium chloride (1%); double distilled water up to 1.0 l. One mL of a filter sterilized solution of kanamycin (4.0 mg/1 mL)

was added to each flask after cooling. Flasks were inoculated with KRC1651 and incubated with shaking at 30 °C. After 24 h, 1.0 mL of toluene was added to the center-well and incubation was continued for one more h. Organisms were harvested and the pellets were washed 2x with phosphate buffer (pH 7.0). Final centrifugation at 17,000 x g for 30 min resulted in 4.34 g wet weight of cells. Center-well flasks containing the PHBA minimal medium in which yeast extract was replaced with 1.0 mM of glucose or 1.0 mM of sodium succinate were inoculated with 620 mg (wet weight) of cells. Varying concentrations of MgCl₂ (1.2 mM - 5.0 mM) were used in these experiments. Toluene (2.0 mL, center-well) was added and the flasks were incubated with shaking at 30 °C. Flasks with standard PHBA trace mineral solutions were sampled at 2, 5, 24, 48 and 68 h. Flasks with increased MgCl₂ concentration were sampled at 2, 48 and 68 h. These were extracted, derivatized and analyzed as described above. The results of these analysis are summarized below in Tables 5 and 6.

TABLE 5

Glucose Medium

<u>Sample</u>	<u>PHBA formed/mL</u>	<u>Sample</u>	<u>PHBA formed/mL</u>
Standard-2	13.8 µg	Increased MgCl ₂ -2	11.6 µg
Increased MgCl ₂	24.16 µg	Increased MgCl ₂ -48	0.08 mg
Standard-24	0.11 mg	Increased MgCl ₂ -68	---
Standard-48	0.44 mg		
Standard-68	0.04 mg		

TABLE 6

Succinate Medium

<u>Sample</u>	<u>PHBA formed</u>	<u>Sample</u>	<u>PHBA formed</u>
Standard-2	32.5 µg	Increased MgCl ₂ -2	9.8 µg
Increased MgCl ₂	38.8 µg	Increased MgCl ₂ -48	26.4 µg
Standard-24	0.07 mg	Increased MgCl ₂ -68	22.48 µg
Standard-48	35.9 µg		
Standard-68	33.64 µg		

As can be seen by the data, PHBA concentrations decreased after 24 h, however no PCA was detected in samples. Further, the higher concentrations of MgCl₂ did not increase the ability of organisms to transform toluene to PHBA.

Conversion of PHBA to PCA by Crude Extracts of KRC1651 and KRC16

Purpose: to determine the ability of *P. mendocina* strains KRC1651 (ATCC 55885) and KRC16 to convert PHBA to PCA. The cells obtained above were disrupted as described before and the crude extract obtained was used for

experiments described above. The reaction mixture contained: NADPH, 6.0 μ moles; PHBA, 11.6 μ moles; FAD, 1.2 μ moles; EDTA, 3.0 μ moles; protein, 2.0 mg; sodium phosphate buffer (50 mM) to final volume of 2.0 mL. To insure that the PCA formed was not further degraded, ethylenediaminetetraacetic acid (EDTA) was used as an inhibitor of protocatechuate dioxygenase. The reaction mixture was incubated with shaking for 30 min at 30 °C after which it was terminated by acidifying to <pH 2 followed by addition of 10 mL ethylacetate. This mixture was then allowed to remain at room temperature for approximately 16 h. It was then further extracted (x2) using 5 mL of ethylacetate. The organic layer was dried over Na₂SO₄ and then evaporated to dryness under a stream of nitrogen. Samples were prepared for GC analysis as before and results are summarized below in Table 7:

TABLE 7

Sample	PHBA Detected	PCA Formed per Reaction
KRC1651	0.88 mg	---
KRC-16	0.86 mg	3.9 μ g

As can be seen by the data, no PCA was detected by GC analysis of the *pobA*(-) strain *P. mendocina* KRC1651 (ATCC 55885). Further, the strain containing the *pobA* gene (*P. mendocina* KRC16) showed formation of 3.9 μ g of PCA indicating that it is converting PHBA to PCA.

EXAMPLE 8Effect of glucose concentration on PHBA production

Example 8 demonstrates the effect of glucose on cell yield and the production of PHBA from KRC1651 (ATCC 55885).

Cell Yield - Fed Batch

Cell yield was tested in the mineral medium with 2 g/L glucose and 0.48 g/L yeast extract. The cell yield obtained was corrected for yeast extract contribution and was the highest on fructose and the lowest on succinate, >0.38 and >0.22 gr cell/gr substrate, respectively. Yeast extract, peptone, casamino acids, tryptone, N-Z-amine E, corn steep liquor, and soytone were tested as organic nitrogen additives to the similar medium. All additives stimulated growth and cell yield. The growth rate was influenced by temperature, medium additions and the density of the culture. The initial growth rates at low cell densities were maximal at 34-36 °C with doubling times of 35 and 33 min. respectively. At higher cell densities, the growth rate was much slower and was estimated in fermenters, at 10 gr cells/L to have a doubling time of about two hours. The final cell densities on lean medium were limited to <4.7 g/L, Fed-Batch studies with

addition of glucose, ammonia, yeast extract and N-Z-amine E achieved 40.0 g/L. Cell yield based on glucose ranged from 0.22 to 0.47 g/g in the lean medium to 0.41 to 0.60 in the rich medium.

Cell Yield - Continuous Culture

- 5 Using glucose as a carbon source, *P. mendocina* demonstrated an ability to grow in continuous culture. Cultures were prepared with 1-L SixFour fermenter with mineral medium, 10 g/L glucose, 0.75 g/L (NH₄)₂SO₄ and 0.28 g/L yeast extract and at dilution rates ranging from 0 to 0.3 h (2.5 h doubling time). The cell yield in these cultures was 0.3 g/g.

10 Production of PHBA in Continuous Culture

The specific productivity of PHBA was tested at 1.25 to 4 g/L cell concentrations and 4 h incubation. Maximal productivity occurred at 0.125-0.5 g/L and it progressively decreased at 1 and 2 g/L.

EXAMPLE 9

15 Effect of pH on PHBA production

The effect of pH was determined on KRC1651 (ATCC 55885) grown in both rich and lean medium according to the conditions recited in the GENERAL METHODS.

- pH had a significant effect on PHBA production, where increasing the pH from 6.3 to 7.4 increased PHBA specific productivity by about two fold. At higher pH values the specific productivity of PHBA varied with the medium used. Lean medium produced no change in PHBA specific productivity in a pH range of 7.2 to 7.9, while in rich medium the specific productivity increased by about 4 fold. The pH of the cultures should be maintained within a range of about from 6.3 to 7.4, where a range from about 7.2 to 7.7 is most preferred.

EXAMPLE 10

Effect of toluene and PHBA concentration on the production of PHBA

- Example 10 demonstrates the effect of toluene concentration on the production of PHBA from KRC1651 (ATCC 55885). Two separate protocols were used for the analysis, one in 125 ml Culture flasks and the other under conditions of batch fermentation in a continuous feed bioreactor.

Culture Flasks

- Growth studies using KRC1651 (ATCC 55885) were performed in 125 mL sealed flasks with 5 mL minimal medium plus 0.28 g/L yeast extract and 10 g/L glucose. The flasks were incubated at 30 °C in incubator shaker. Growth was observed after 24 and 48 h visually or by OD measurement at 600 nm.

Mutants with higher tolerance to toluene and PHBA were selected by repeated transfers in media with increasing concentrations of toluene and PHBA.

Repeated transfers produced strains that could grow on agar plates at 40 g/L PHBA and about 100 ppm toluene.

- Both toluene and PHBA have a bacteriostatic and bacteriocitic effects. The growth of KRC1651 was significantly inhibited at about 100 ppm toluene or 10 g/L PHBA. Together these agents had an additive toxic effect.

Bioreactor

- KRC1651 (ATCC 55885) was used in bioreactor studies and grown in rich and lean medium. Production of PHBA from toluene can be enhanced by a continuous feed of toluene to a bioreactor to keep the steady state concentration of toluene in the reactor below toluene explosivity limits. The lower explosivity limits (LEL) for toluene is 120 ppm in the liquid phase, 1.2% v/v in the gas phase. Glucose was fed as a co-substrate for cell maintenance simultaneously with toluene feed. The standard system used consisted of 1 mM glucose, 60 ppm toluene, 0.25-0.5 g cells/L, and the incubation was for 3-4 h. The pH was controlled at 7.2 with addition of buffer. The effect of toluene concentration on PHBA production was examined at 30, 60, and 100 ppm.

- The time course of PHBA production was followed from 0 to 24 h. PHBA production (based on accumulation) demonstrated a lag of 1 to 2 h where maximal productivity was at about 2-3 h. At later times a decline from 4 to 6-10 h and zero production from 6-10 h to 24 h was seen. No decline in PHBA concentration was observed. Results were comparable between rich and lean medium; however, in the rich medium the PHBA productivity was somewhat higher reaching a maximum of 60 ppm.

EXAMPLE 11

- Production of PHBA with *pobA* (-) *P. mendocina* - Effect of Mg+ on Toluene Tolerance

- Toluene and PHBA are known to incorporate and interfere with the integrity of bacterial membranes and function. Growth studies in the presence of inhibitory concentrations of toluene and PHBA were used to assess the protective effect of Mg ions.

- Experimental: Growth studies using KRC1651 (ATCC 55885) were performed in 125 mL sealed flasks with 5 mL minimal medium plus 0.28 g/L yeast extract and 10 g/L glucose. The flasks were incubated at 30 °C in incubator shaker. Growth was observed after 24 and 48 h visually or by OD measurement at 600 nm.

Results: Magnesium ions increased KRC1651 tolerance to toluene and PHBA. Increasing Mg concentration from 2 to 12 mM enhances growth rate at 100 and 150 ppm toluene and allows growth at 100 ppm toluene and 5 g/L PHBA.

EXAMPLE 12

Maximal production rates of PHBA

KRC1651 (ATCC 55885) was cultured in rich medium where the growth conditions of pH, glucose, Mg ion concentration and toluene were optimized for maximal PHBA production as described in Examples 8-11. With all conditions optimized, PHBA production varied with the method of production. The most apparent difference was between cells that grew in fermenters and flasks. Cells that grew in fermenters had an average specific productivity of 0.35 g/g-h and cells that grew in flasks had an average specific productivity of 0.11 g/g-h. The maximal specific productivity in fermenters occurs at 1-3 h and is estimated to be 1 g/g-h.

EXAMPLE 13

Fed-Batch Fermentation of Toluene to PHBA

A two-step fermentation was carried out to demonstrate the production of PHBA from toluene using the *P. mendocina* KRC16KDpobA51 strain. This strain had disrupted *pobA-1* and *pobA-2* genes. The fermentation was carried out in two steps:

1. biomass built-up step (batch process), and
2. biotransformation step (fed-batch process)

During the first step, the *P. mendocina* KRC16KDpobA51 strain grew to high cell densities in a 5 L fermenter with approximately a 3 L working volume. The medium contained 1.2 g KH_2PO_4 , 6 g $(\text{NH}_4)_2\text{SO}_4$, 0.3 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20 mL/L trace elements, 20 g/L glucose, 20 g/L corn steep liquor, 1 mL/L polypropylene glycol and 1 g/L yeast extract. The trace elements contained 10 g/L citric acid, 1.5 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2.8 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.39 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.38 g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.2 g/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, and 0.3 g/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$. The phosphate, ammonia, Mg salts and trace elements were sterilized together and then titrated to pH 7.2 with KOH or H_2SO_4 before addition of the remaining components.

The second step commenced as a toluene fed-batch fermentation using glucose as co-feed at a rate of 3 g/h. The rate was decreased if glucose concentration rose above 1 g/L. Toluene was supplied at a rate that kept its concentration in the medium at 10-100 ppm. The initial cell density was 16.2 OD units. Over 18.5 h, a total of 67.3 g (0.73 moles) of toluene was fed during step 2 of the biotransformation. All of the toluene was not utilized and some was lost with the air exit-stream of the fermenter. After 18.5 h, a total of 38.1 g PHBA (0.278 moles) was produced. Table 8 summarizes the production of PHBA over time.

TABLE 8

Time (h)	PHBA (g/L)
0.0	0.0
1.0	0.0
1.4	0.0
2.4	0.7
3.4	1.7
4.5	3.4
5.4	6.1
6.4	7.6
7.3	6.9
8.3	10.7
9.3	9.1
10.5	8.9
11.5	9.1
12.7	10.7
14.5	9.7
15.5	12.1
17.0	11.4
17.4	12.6
18.5	12.6
18.5	12.7

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT:
 - (A) ADDRESSEE: E. I. DU PONT DE NEMOURS AND COMPANY
 - (B) STREET: 1007 MARKET STREET
 - (C) CITY: WILMINGTON
 - (D) STATE: DELAWARE
 - (E) COUNTRY: U.S.A.
 - (F) ZIP: 19898
 - (G) TELEPHONE: 302-982-8112
 - (H) TELEFAX: 302-773-0164
 - (I) TELEX: 6717325
- (ii) TITLE OF INVENTION: METHOD FOR THE PRODUCTION OF
PARA-HYDROXYBENZOATE IN
PSEUDOMONAS MENDOCINA
- (iii) NUMBER OF SEQUENCES: 10
- (iv) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: DISKETTE, 3.50 INCH
 - (B) COMPUTER: IBM PC COMPATIBLE
 - (C) OPERATING SYSTEM: MICROSOFT WINDOWS 95
 - (D) SOFTWARE: MICROSOFT WORD VERSION 7.0A
- (v) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vi) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 60/049,556
 - (B) FILING DATE: JUNE 13, 1997
- (vii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: FLOYD, LINDA A.
 - (B) REGISTRATION NUMBER: 33,692
 - (C) REFERENCE/DOCKET NUMBER: CR-9967-A

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1140 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```

ATGAATACTC AAGTTGCTAT CATCGGCGCC GGTCTTCGG GGCTTCTGCT CGGCCAACTG   60
CTGCACAAGG CCGGTATCGA AACCATCATC CTCGAGCGGC AGACTCCCGA CTATGTGCTC  120
GGCCGTATCC GGGCCGGCGT GCTGGAGCAG GGCACCGTCG ATATGTTGCG CGAAGCCGGT  180
GTTGCGCAAC GTATGGATGC CGAGGGGCTG GTCCATGAGG GGGTCGAGCT GGTGTTTCGAC  240
GGCAAGCGCG TGCCGATTCA TCTGAAGGCA TTGACCGGCG GCAAGACCGT GATGGTTTAT  300
GGCCAGACTG AAGTCACCCG TGACCTGATG GATGCCCGCG TCGCTGTCGG CGCGCCCATC  360
GTGTACTCCG CCGAGAACGT GCAGCTGCAC GAACTCAAGG GCGGCAAGCC TTACGTCACC  420
TTCGAGAAGG ATGGCCAATC CCATCGCATC GATTGCGACT ACATCGCCGG TTGCGATGGC  480
TTCCATGGCA TCGCGCGCAA GAGCATTCGG GCCGGTGTCC TGACCGAGTA CGAGCGTGTC  540
TACCCGTTTCG GCTGGCTGGG CCTGTTGTCA GACACCCCGC CGGTCCACGA GGAAGTGATC  600
TACGCCACAC ACGAGCGCGG TTTCGTGCTG TGCAGCCAGC GTTCGCTGAC CCGCAGTCGC  660
TATTACTTGC AGGTTCCGCT GACCGACAAA GTCGAAGACT GGTCCGATGA GCGTTTCTGG  720
AACGAGCTCA AGGCCCGTCT TCCGCAGGAC GTGGCGGACA GGCTGGTCAC CGGCCCGTCC  780
CTGGAAAAGA GTATCGCCCC GCTGCGAAGC TTCGTGGTCG AACCGATGCA GTACGGCAAC  840
CTGTTTCCTAG TGGGGGACGC CGCCCATATC GTGCCGCCGA CGGGGGCCAA GGGCCTAAAC  900
CTGGCCGCCT CCGACGTATG CTACCTGTAT CGAATCCTGA TCAAGGTCTA CAAGGAAGGT  960
CGCACCGACC TGCTGGAGAA GTATTCGGAG CTGGCTCTAC GCCGCGTGTG GAAAGGTGAA 1020
CGCTTCAGCT GGTTTCATGAC CAATCTGCTG CACGACTTCG ACGGTCACCA GGACGCCTGG 1080
GATCAGAAGA TGCAGAAAGC CGACCGCGAG TACTACCTGA AGTCCCACGC TGGTCTGGCC 1140

```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "PRIMER"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GRNATRCTYC TYTANCTYAT C

21

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1188 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```

ATGAAAACCC AGGTTGCCAT TATCGGTGCC GGTCCGTCCG GCCTGCTGCT CGGTCAATTG   60
CTGCACAAGG CGGGTATCGA CACCCATCATC GTCGAGCGCC AGACCCCTGA GTACGTGCTC  120
GGCCGCATCC GCGCCGGCGT GCTGGAACAG GGCACCGTGG ACATGCTCCG CGAGGCGGGC  180
GTGAGCGAGC GCATGGACGC CGAGGGCCTG GTACACCACG GCGTCGAGCT GGTGTTTCGAT  240
GGCAAGCGGG TGCCGGTCCA CCTTACCGAG CTGACCGGTG GCAAGAGCGT GATGGTCTAT  300
GGCCAGACCG AAGTCACCCG CGACCTGATG GACGCCCGGG CCGCCAGCGG CGCCCCCATC  360
GTCTACTCCG CCAGCAACGT GGAGCTGCAT GAACTCAAGG GCGAGCGCCC CTACGTGACC  420
TTCGAGAAGG ACGGCGAGCG GGTGCGCGTC GACTGCGACT ACATCGCCGG TTGCGACGGC  480
TTCCATGGCG TGTCGCGCAA GAGCATCCCG GAAGGCGTGC TGACCGAGTA CGAGCGCGTC  540
TATCCCTTCG GCTGGCTGGG CCTGCTCTCC GACACCCCGC CGGTGCATGA GGAATCATC  600
TACGCCACC ACGAGCGCGG CTTCTCCCTG TGCAGCCAGC GTTCCCTCAC CCGCAGCCGC  660
TACTACTTGC AGGTGCCGCT GACCGACAAG GTGGAGGACT GGTCCGACGA GCGCTTCTGG  720
AACGAGCTGA AGGCGGTCT GCCCGAGGAC GTGGCGGCCA AGCTGGTGAC CGGCCCGTCC  780
CTGGAGAAGA GCATCGCGCC CCTGCGCAGC TTCGTGGTCG AGCCCATGCA GTACGGCCAT  840
CTGTTCTCTG TGGGTGATGC CGCCACATC GTGCCGCCCA CCGGCGCCAA GGGCCTGAAC  900
CTGGCGGCTT CCGACGTCTG CTACCTCTAT CGCATCCTGG TGAAGGTGTA CCGCGAAGGC  960
CGTACCGACC TGCTGGAGAA ATACTCCGAA CTGGCCCTGC GCCGGGTGTG GAAAGGCGAG 1020
CGCTTCAGCT GGTTTCATGAC CAACCTGCTG CACGACTTCG ACGGCCACAA GGACGCCTGG 1080
GACCAGAAGA TGCAGCAGGC CGACCGCGAG TACTTCCTCG GTTCCCACGC GGGCCTCGTG 1140
AACATCGCCG AGAACTACGT GGGCCTGCCC TACGAAGACG TGAAATAA           1188
  
```

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 268 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

Met Asn Thr Gln Val Ala Ile Ile Gly Ala Gly Pro Ser Gly Leu Leu
1           5           10           15
  
```

```

Leu Gly Gln Leu Leu His Lys Ala Gly Ile Glu Thr Ile Ile Leu Glu
      20              25              30
Arg Gln Thr Pro Asp Tyr Val Leu Gly Arg Ile Arg Ala Gly Val Leu
      35              40              45
Glu Gln Gly Thr Val Asp Met Leu Arg Glu Ala Gly Val Ala Gln Arg
      50              55              60
Met Asp Ala Glu Gly Leu Val His Glu Gly Val Glu Leu Val Phe Asp
      65              70              75              80
Gly Lys Arg Val Pro Ile His Leu Lys Ala Leu Thr Xaa Xaa Xaa Xaa
      85              90              95
Xaa Xaa Gly Gly Lys Thr Val Met Val Tyr Gly Gln Thr Glu Val Thr
      100             105             110
Arg Asp Leu Met Asp Ala Arg Val Ala Val Gly Ala Pro Ile Val Tyr
      115             120             125
Ser Ala Glu Asn Val Gln Leu His Glu Leu Lys Gly Gly Lys Pro Tyr
      130             135             140
Val Thr Phe Glu Lys Asp Gly Gln Ser His Arg Ile Asp Cys Asp Tyr
      145             150             155             160
Ile Ala Gly Cys Asp Gly Phe His Gly Ile Ala Arg Lys Ser Ile Pro
      165             170             175
Ala Gly Val Leu Thr Glu Tyr Glu Arg Val Tyr Pro Phe Gly Trp Leu
      180             185             190
Gly Leu Leu Ser Asp Thr Pro Pro Val His Glu Glu Leu Ile Tyr Ala
      195             200             205
His His Glu Arg Gly Phe Val Leu Cys Ser Gln Arg Ser Leu Thr Arg
      210             215             220
Ser Arg Tyr Tyr Leu Gln Val Pro Leu Thr Asp Lys Val Glu Asp Trp
      225             230             235             240
Ser Asp Glu Arg Phe Trp Asn Glu Leu Lys Ala Arg Leu Pro Gln Asp
      245             250             255
Val Ala Asp Arg Leu Val Thr Gly Pro Ser Leu Glu
      260             265

```

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 395 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: not relevant
 - (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```

Met Lys Thr Gln Val Ala Ile Ile Gly Ala Gly Pro Ser Gly Leu Leu
1          5          10          15
Leu Gly Gln Leu Leu His Lys Ala Gly Ile Asp Thr Leu Ile Val Glu
      20              25              30

```

Arg Gln Thr Pro Glu Tyr Val Leu Gly Arg Ile Arg Ala Gly Val Leu
 35 40 45
 Glu Gln Gly Thr Val Asp Met Leu Arg Glu Ala Gly Val Ser Glu Arg
 50 55 60
 Met Asp Ala Glu Gly Leu Val His His Gly Val Glu Leu Val Phe Asp
 65 70 75 80
 Gly Lys Arg Val Pro Val His Leu Thr Glu Leu Thr Gly Gly Lys Ser
 85 90 95
 Val Met Val Tyr Gly Gln Thr Glu Val Thr Arg Asp Leu Met Asp Ala
 100 105 110
 Arg Ala Ala Ser Gly Ala Pro Ile Val Tyr Ser Ala Ser Asn Val Glu
 115 120 125
 Leu His Glu Leu Lys Gly Glu Arg Pro Tyr Val Thr Phe Glu Lys Asp
 130 135 140
 Gly Glu Arg Val Arg Val Asp Cys Asp Tyr Ile Ala Gly Cys Asp Gly
 145 150 155 160
 Phe His Gly Val Ser Arg Lys Ser Ile Pro Glu Gly Val Leu Thr Glu
 165 170 175
 Tyr Glu Arg Val Tyr Pro Phe Gly Trp Leu Gly Leu Leu Ser Asp Thr
 180 185 190
 Pro Pro Val His Glu Glu Leu Ile Tyr Ala His His Glu Arg Gly Phe
 195 200 205
 Ser Leu Cys Ser Gln Arg Ser Leu Thr Arg Ser Arg Tyr Tyr Leu Gln
 210 215 220
 Val Pro Leu Thr Asp Lys Val Glu Asp Trp Ser Asp Glu Arg Phe Trp
 225 230 235 240
 Asn Glu Leu Lys Ala Arg Leu Pro Glu Asp Val Ala Ala Lys Leu Val
 245 250 255
 Thr Gly Pro Ser Leu Glu Lys Ser Ile Ala Pro Leu Arg Ser Phe Val
 260 265 270
 Val Glu Pro Met Gln Tyr Gly His Leu Phe Leu Val Gly Asp Ala Ala
 275 280 285
 His Ile Val Pro Pro Thr Gly Ala Lys Gly Leu Asn Leu Ala Ala Ser
 290 295 300
 Asp Val Cys Tyr Leu Tyr Arg Ile Leu Val Lys Val Tyr Arg Glu Gly
 305 310 315 320
 Arg Thr Asp Leu Leu Glu Lys Tyr Ser Glu Leu Ala Leu Arg Arg Val
 325 330 335
 Trp Lys Gly Glu Arg Phe Ser Trp Phe Met Thr Asn Leu Leu His Asp
 340 345 350
 Phe Asp Gly His Lys Asp Ala Trp Asp Gln Lys Met Gln Gln Ala Asp
 355 360 365
 Arg Glu Tyr Phe Leu Gly Ser His Ala Gly Leu Val Asn Ile Ala Glu
 370 375 380

Asn Tyr Val Gly Leu Pro Tyr Glu Asp Val Lys
 385 390 395

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1446 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GATCAGCGCC GGCGCATGCG CCGCCGGCCA GCCATAACAG CGAGAGGGCT ATCCGCAATG 60
 AAGACTCAAG TCGCCATCAT CGGCGCCGGT CCGTCCGGCC TCCTGCTCGG CCAGTTGCTG 120
 CACAAGGCCG GCATCGACAA CGTGATCCTC GAACGCCAGA CCCC GGACTA CGTGCTCGGC 180
 CGCATCCGCG CCGGCGTGCT GGAACAGGGT ATGGTCGACC TGCTGCGCGA GGCCGGCGTC 240
 GACCGGCGCA TGGCGCGCGA CGGGCTGGTC CACGAAGGCG TGGAGATCGC CTTCGCCGGG 300
 CAGCGCCGGC GCATCGACCT GAAGCGCCTG AGCGGCGGCA AGACGGTGAC GGTCTACGGC 360
 CAGACCGAGG TCACCCGCGA CCTCATGGAA GCCGCGAAG CCTGCGGCGC CACTACCGTC 420
 TACCAGGCCG CCGAGGTGCG CCTGCACGAC CTGCAAGGTG AGCGCCCCTA CGTGACCTTC 480
 GAACGCGACG GCGAACGGCT ACGCCTGGAT TGCGACTACA TCGCCGGCTG CGATGGCTTC 540
 CACGGCATCT CGCGGCAATC GATCCCGGCG GAGCGGCTGA AGGTCTTCGA GCGGGTCTAT 600
 CCGTTGCGCT GGCTCGGCCT GCTCGCCGAC ACCCCGCCGG TCAGCCACGA ACTGATCTAC 660
 GCCAACCATC CGCGCGGCTT CGCCCTGTGC AGCCAGCGTT CGGCGACCCG CAGCCGCTAC 720
 TACGTACAGG TGCCATTGAC AGAGAAGGTC GAGGACTGGT CCGACGAGCG CTTCTGGACG 780
 GAACTGAAAG CGCGCCTCCC GGCCGAGGTG GCGGAGAAAC TGGTGACCGG TCCTTCGCTG 840
 GAGAAGAGCA TCGCGCCGCT GCGCAGCTTC GTGGTCGAGC CGATGCAGCA TGGCCGGCTG 900
 TTCCTCGCCG GCGACGCCGC GCACATCGTG CCGCCCACCG GCGCCAAGGG ACTGAACCTG 960
 GCGGCCAGCG ACGTCAGCAC GCTCTACCGG CTGCTGCTGA AGGCCTACCG CGAAGGGCGG 1020
 GGCGAAGTGC TGGAACGCTA CTCGGCAATC TGCCTGCGGC GGATCTGGAA GGCCGAACGC 1080
 TTCTCCTGGT GGATGACTTC GGTGCTGCAT CGCTTCCCCG ACACCGACGC GTTCAGCCAG 1140
 CGCATCCAGC AGACCGAACT GGAGTACTAC CTGGGCTCCG AGGCGGGCCT GGCGACCATC 1200
 GCCGAGAACT ATGTCGGCCT GCCCTACGAG GAAATCGAGT AGGCCGCGCC ACCCCGGCGC 1260
 AACGCCGGGG CACCGCTCCC AAGGCGGGAC GAACGTTGGC GCAGGCGAAT ATCGACGAAA 1320
 GACGACCCTC GGACTTCGAT ATTCTCCGGC ACCTGTCCAT CCCCAGGCGC AGTATGATGC 1380
 CCGTGCCTTC GCGTTCCGTC CGCCAGGTG CCGCTCTTGT CCGATCTGCC AGAACCCGCC 1440
 CGCCGT 1446

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1315 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```

AGGCTTTGTC GCAGTTTTC CTTCTTATG CTGCCCCTC AAGTGAAAAA TGTCAGCCAT   60
AACACCAATG ACAACTCTTT CACTTGATCC GTTCGGGTGA AGCTTGCGAG CCATAAAAAT  120
AATGAAAACG CTAAAAACCC AAGTCGCCAT TATTGGCGCC GGTCCCTCCG GATTGCTGCT  180
CGGCCAGTTA CTGCACAACG CGGGTATCCA GACCCTGCTT CTAGAGCGCC AGAGCGCCGA  240
CTACGTGCAA GGCCGCATCC GTGCCGGGGT GCTGGAGCAA GGCATGGTCG ACCTGCTGCG  300
CGAAGCGGGC GTCAGCCGAC GCATGGACGC CGAGGGCCTT GTGCATGACG GTTTCGAATT  360
GGCACTCAAT GGCGAACTCA CCCACATCGA CCTCAAGGCG CTCACCGGCG GCCAGTCGGT  420
GATGATCTAC GGCCAGACCG AAGTACCCG TGAATTGATG GCCGCCCGCG AAGCGGCGGG  480
TGGCATCACT CTATACGAAA CGCAGAACGT GCAGCCTCAT GGTACAAAAA CTGATCGACC  540
CTGGCTGACC TTCGAGCACC AGGGTGAAGC TTTTCGCCTG GAGTGCGACT ACATCGCGGG  600
CTGTGATGGT TTTCACGGTG TGGCGCGGCA GTCGATTCCG GCGCAGTCGT TGAAGTCTT  660
CGAGCGCGTC TATCCCTTCG GTTGGCTGGG CGTCCTCGCC GACACACCGC CGGTGCATGA  720
CGAACTGGTG TACGCCAAAC ATGCGCGTGG CTTTGCCCTG TGCAGCATGC GCTCGCCGAC  780
CCGCAGCCGC TATTACCTGC AAGTGCCGGT TGAAGAAGCG CTGGATGAAT GGTCGGATCA  840
GCGCTTCTGG GATGAGCTGA AAACCCGTTT GCCCAGTGCA CTGGCGGCCC AACTGGTCAC  900
CGGGCCATCC ATCGAGAAGA GCATCGCGCC CGCTGCGAGC TTTGAGGTCG AGCCGATGCA  960
ATACGGGCGC CTGTTCTTGC TGGGGGACGC CGCGCATATC GTGCCGCCCA CCGGGGCCAA 1020
GGGCTTGAAC CTGGCGGCCA GCGACGTGAG TACGCTGTTT CGGATCTTGC TCAAGGTCTA 1080
TCGCGAGGGG CGGGTGGACC TGCTGGAACA GTACTCAGCG ATCTGCTTGC GCCGCGTATG 1140
GAAAGCCGAA CGTTTTTCCT GGTGGATGAC TTCGATGTTG CACCAGTTTC CGGAGGCCGA 1200
CGGGTTCAGC CAGCGCATTG CCGAGAGCGA GCTTGCGTAT TTCATCAGCT CCGAGGCGGG 1260
CCGCAAAACC ATCGCAGAAA ATTACGTCGG GCTTCCTTAC GAAGCTATCG AGTAG      1315

```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 394 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Lys Thr Gln Val Ala Ile Ile Gly Ala Gly Pro Ser Gly Leu Leu
 1 5 10 15
 Leu Gly Gln Leu Leu His Lys Ala Gly Ile Asp Asn Val Ile Leu Glu
 20 25 30
 Arg Gln Thr Pro Asp Tyr Val Leu Gly Arg Ile Arg Ala Gly Val Leu
 35 40 45
 Glu Gln Gly Met Val Asp Leu Leu Arg Glu Ala Gly Val Asp Arg Arg
 50 55 60
 Met Ala Arg Asp Gly Leu Val His Glu Gly Val Glu Ile Ala Phe Ala
 65 70 75 80
 Gly Gln Arg Arg Arg Ile Asp Leu Lys Arg Leu Ser Gly Gly Lys Thr
 85 90 95
 Val Thr Val Tyr Gly Gln Thr Glu Val Thr Arg Asp Leu Met Glu Ala
 100 105 110
 Arg Glu Ala Cys Gly Ala Thr Thr Val Tyr Gln Ala Ala Glu Val Arg
 115 120 125
 Leu His Asp Leu Gln Gly Glu Arg Pro Tyr Val Thr Phe Glu Arg Asp
 130 135 140
 Gly Glu Arg Leu Arg Leu Asp Cys Asp Tyr Ile Ala Gly Cys Asp Gly
 145 150 155 160
 Phe His Gly Ile Ser Arg Gln Ser Ile Pro Ala Glu Arg Leu Lys Val
 165 170 175
 Phe Glu Arg Val Tyr Pro Phe Gly Trp Leu Gly Leu Leu Ala Asp Thr
 180 185 190
 Pro Pro Val Ser His Glu Leu Ile Tyr Ala Asn His Pro Arg Gly Phe
 195 200 205
 Ala Leu Cys Ser Gln Arg Ser Ala Thr Arg Ser Arg Tyr Tyr Val Gln
 210 215 220
 Val Pro Leu Thr Glu Lys Val Glu Asp Trp Ser Asp Glu Arg Phe Trp
 225 230 235 240
 Thr Glu Leu Lys Ala Arg Leu Pro Ala Glu Val Ala Glu Lys Leu Val
 245 250 255
 Thr Gly Pro Ser Leu Glu Lys Ser Ile Ala Pro Leu Arg Ser Phe Val
 260 265 270
 Val Glu Pro Met Gln His Gly Arg Leu Phe Leu Ala Gly Asp Ala Ala
 275 280 285
 His Ile Val Pro Pro Thr Gly Ala Lys Gly Leu Asn Leu Ala Ala Ser
 290 295 300
 Asp Val Ser Thr Leu Tyr Arg Leu Leu Leu Lys Ala Tyr Arg Glu Gly
 305 310 315 320
 Arg Gly Glu Leu Leu Glu Arg Tyr Ser Ala Ile Cys Leu Arg Arg Ile
 325 330 335

Trp	Lys	Ala	Glu 340	Arg	Phe	Ser	Trp	Trp 345	Met	Thr	Ser	Val	Leu 350	His	Arg
Phe	Pro	Asp 355	Thr	Asp	Ala	Phe	Ser 360	Gln	Arg	Ile	Gln	Gln 365	Thr	Glu	Leu
Glu	Tyr 370	Tyr	Leu	Gly	Ser	Glu 375	Ala	Gly	Leu	Ala	Thr 380	Ile	Ala	Glu	Asn
Tyr 385	Val	Gly	Leu	Pro	Tyr 390	Glu	Glu	Ile	Glu						

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 397 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Met 1	Lys	Thr	Leu	Lys 5	Thr	Gln	Val	Ala	Ile 10	Ile	Gly	Ala	Gly	Pro 15	Ser
Gly	Leu	Leu	Leu 20	Gly	Gln	Leu	Leu	His 25	Asn	Ala	Gly	Ile	Gln 30	Thr	Leu
Leu	Leu	Glu 35	Arg	Gln	Ser	Ala	Asp 40	Tyr	Val	Gln	Gly	Arg 45	Ile	Arg	Ala
Gly	Val 50	Leu	Glu	Gln	Gly	Met 55	Val	Asp	Leu	Leu	Arg 60	Glu	Ala	Gly	Val
Ser 65	Arg	Arg	Met	Asp 70	Ala	Glu	Gly	Leu	Val	His 75	Asp	Gly	Phe	Glu	Leu 80
Ala	Leu	Asn	Gly	Glu 85	Leu	Thr	His	Ile	Asp 90	Leu	Lys	Ala	Leu	Thr 95	Gly
Gly	Gln	Ser	Val 100	Met	Ile	Tyr	Gly	Gln 105	Thr	Glu	Val	Thr	Arg 110	Asp	Leu
Met	Ala	Ala 115	Arg	Glu	Ala	Ala	Gly 120	Gly	Ile	Thr	Leu	Tyr 125	Glu	Thr	Gln
Asn	Val 130	Gln	Pro	His	Gly	His 135	Lys	Thr	Asp	Arg	Pro 140	Trp	Leu	Thr	Phe
Glu 145	His	Gln	Gly	Glu	Ala 150	Phe	Arg	Leu	Glu	Cys 155	Asp	Tyr	Ile	Ala	Gly 160
Cys	Asp	Gly	Phe	His 165	Gly	Val	Ala	Arg	Gln 170	Ser	Ile	Pro	Ala	Gln 175	Ser
Leu	Lys	Val	Phe 180	Glu	Arg	Val	Tyr	Pro 185	Phe	Gly	Trp	Leu	Gly 190	Val	Leu
Ala	Asp	Thr 195	Pro	Pro	Val	His	Asp 200	Glu	Leu	Val	Tyr	Ala 205	Lys	His	Ala
Arg	Gly 210	Phe	Ala	Leu	Cys	Ser 215	Met	Arg	Ser	Pro	Thr 220	Arg	Ser	Arg	Tyr

Tyr Leu Gln Val Pro Val Glu Glu Ala Leu Asp Glu Trp Ser Asp Gln
 225 230 235 240
 Arg Phe Trp Asp Glu Leu Lys Thr Arg Leu Pro Ser Ala Leu Ala Ala
 245 250 255
 Gln Leu Val Thr Gly Pro Ser Ile Glu Lys Ser Ile Ala Pro Ala Ala
 260 265 270
 Ser Phe Glu Val Glu Pro Met Gln Tyr Gly Arg Leu Phe Leu Leu Gly
 275 280 285
 Asp Ala Ala His Ile Val Pro Pro Thr Gly Ala Lys Gly Leu Asn Leu
 290 295 300
 Ala Ala Ser Asp Val Ser Thr Leu Phe Arg Ile Leu Leu Lys Val Tyr
 305 310 315 320
 Arg Glu Gly Arg Val Asp Leu Leu Glu Gln Tyr Ser Ala Ile Cys Leu
 325 330 335
 Arg Arg Val Trp Lys Ala Glu Arg Phe Ser Trp Trp Met Thr Ser Met
 340 345 350
 Leu His Gln Phe Pro Glu Ala Asp Gly Phe Ser Gln Arg Ile Ala Glu
 355 360 365
 Ser Glu Leu Ala Tyr Phe Ile Ser Ser Glu Ala Gly Arg Lys Thr Ile
 370 375 380
 Ala Glu Asn Tyr Val Gly Leu Pro Tyr Glu Ala Ile Glu
 385 390 395

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "PRIMER"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CARTTRCTNC AYAANGTNGG NAT

23

Applicant's or agent's file reference number	CR9967A	International application No.	Unknown
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>3</u> , line s <u>29 - 32</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (including postal code and country) 10801 University Blvd. Manassas, Virginia 20110-2209 USA	
Date of deposit 26 November 1996	Accession Number 55885
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)	
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>3</u> , line s <u>33 - 36</u>	
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Address of depositary institution (including postal code and country) 10801 University Blvd. Manassas, Virginia 20110-2209 USA	
Date of deposit 26 November 1996	Accession Number 55886
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WHAT IS CLAIMED IS:

1. A method for the production of PHBA comprising:
 - (i) culturing a *pobA*(-) *Pseudomonas* strain in a medium containing an aromatic organic substrate, at least one suitable carbon source, and a nitrogen source, wherein the *pobA*(-) *Pseudomonas* strain comprises *pobA* genes encoding the TMO toluene degradation pathway and wherein the *pobA*(-) *Pseudomonas* strain does not produce any detectable *para*-hydroxybenzoate hydroxylase, whereby PHBA accumulates; and
 - (ii) recovering the PHBA.
2. The method of Claim 1 wherein the genes encoding the TMO toluene degradation pathway of the *pobA*(-) *Pseudomonas* strain are characterized by a disruption in at least two *pobA* genes.
3. The method of Claim 1 wherein the *pobA*(-) *Pseudomonas* strain is selected from the group consisting of KRC1651 (ATCC 55885), KRC1616, and KRC1615.
4. The method of Claim 2 wherein at least one *pobA* gene encodes a *para*-hydroxybenzoate hydroxylase enzyme having a first amino acid sequence as set out in SEQ ID NO:3 or a second amino acid sequence as set out in SEQ ID NO:4 and wherein the first and second amino acid sequence may encompass amino acid substitutions, deletions or additions that do not alter the function of the *para*-hydroxybenzoate hydroxylase.
5. The method of Claim 2 wherein at least one *pobA* gene is *pobA-1* and is defined by SEQ ID NO:1.
6. The method of Claim 2 wherein at least one *pobA* gene is *pobA-2* and is defined by SEQ ID NO:2.
7. The method of Claim 1 wherein the aromatic organic substrate is selected from the group consisting of toluene, *p*-cresol, *p*-hydroxybenzyl alcohol, benzoate and *p*-hydroxybenzaldehyde.
8. The method of Claim 7 wherein toluene is present in the medium in a concentration of less than 500 ppm.
9. The method of Claim 7 wherein toluene is present in the medium from about 30 ppm to about 60 ppm.
10. The method of Claim 1 wherein the suitable carbon source is succinate, lactate, acetate, ethanol, monosaccharides, oligosaccharides, polysaccharides, or mixtures thereof.
11. The method of Claim 10 wherein the suitable carbon source is glucose or succinate.
12. The method of Claim 1 wherein PHBA accumulates at a concentration of at least 1 mM.

13. The method of Claim 1 wherein PHBA accumulates at a rate of about 0.01 g PHBA/g-cells-h to about 1 g PHBA/g-cells-h.

14. The method of Claim 1 wherein the *pobA*(-) *Pseudomonas* strain is cultured at a pH of from about 6.3 to about 7.9.

5 15. The method of Claim 1, the medium further containing a Mg ion at a concentration of about 2 mM to about 12 mM.

16. A *pobA* gene encoding a *para*-hydroxybenzoate hydroxylase enzyme having the amino acid sequence as shown by SEQ ID NO:3 or SEQ ID NO:4.

10 17. The gene of Claim 16 wherein the gene is *pobA-1* and is defined by SEQ ID NO:1 or is *pobA-2* and is defined by SEQ ID NO:2.

18. A transformed *pobA*(-) *Pseudomonas* strain comprising an amino acid disruption in at least two *pobA* genes.

15 19. The transformed *pobA*(-) *Pseudomonas* strain of Claim 18 selected from the group consisting of KRC1651 (ATCC 55885), KRC1616, and KRC1615.

20. A *Pseudomonas* strain selected from the group consisting of biological deposits identified as KRC1651 (ATCC 55885) and KRC16 (ATCC 55886).

20 21. A TMO *P. mendocina* variant having a cell density at least 6 fold greater than the wildtype under growth conditions of approximately 160ppm toluene, the TMO *P. mendocina* variant produced by mutagenesis of the wildtype KR-1.

22. The TMO *P. mendocina* variant of Claim 21 selected from the group consisting of KRC16 and KRC50.

FIG. 1a

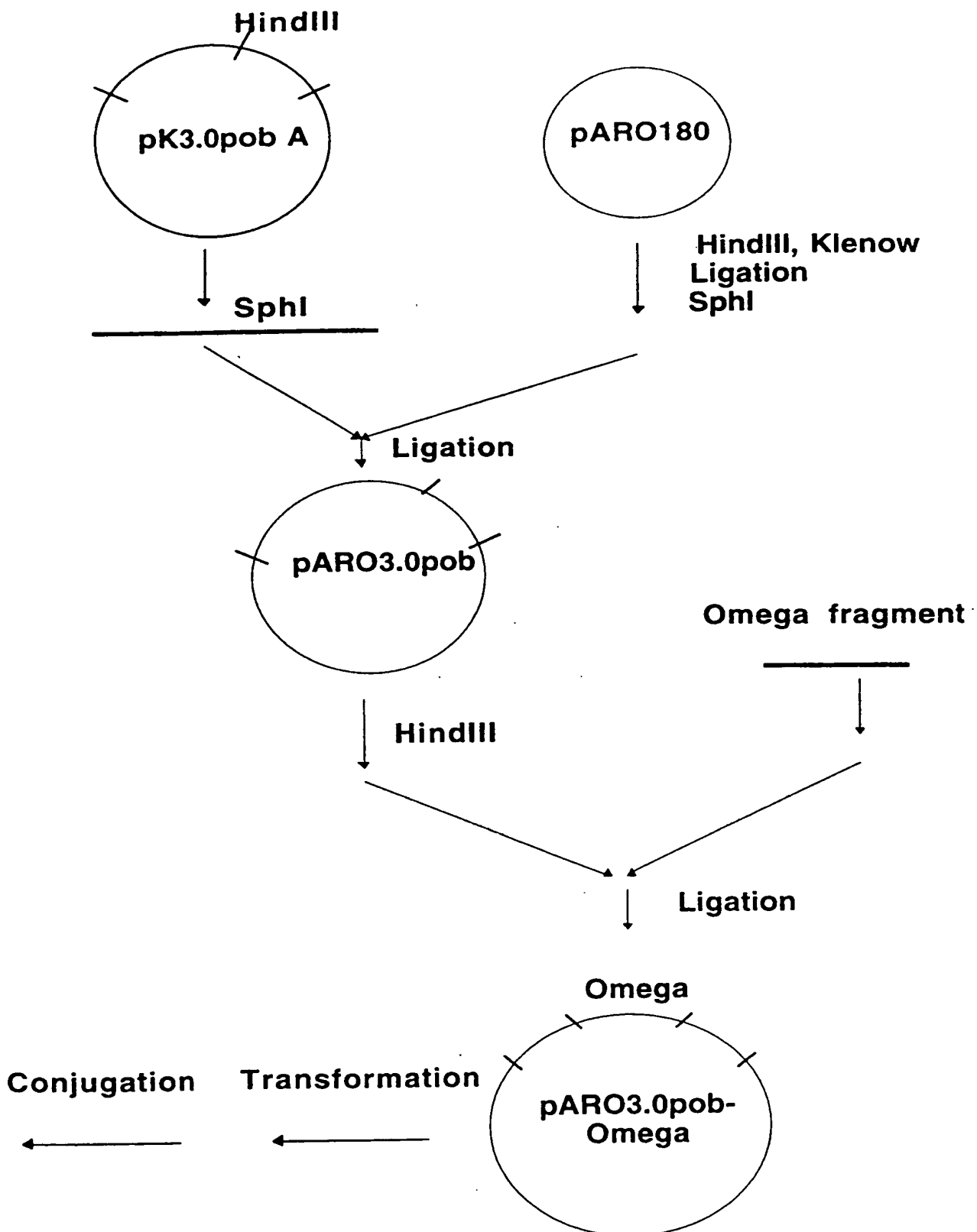


FIG. 1b

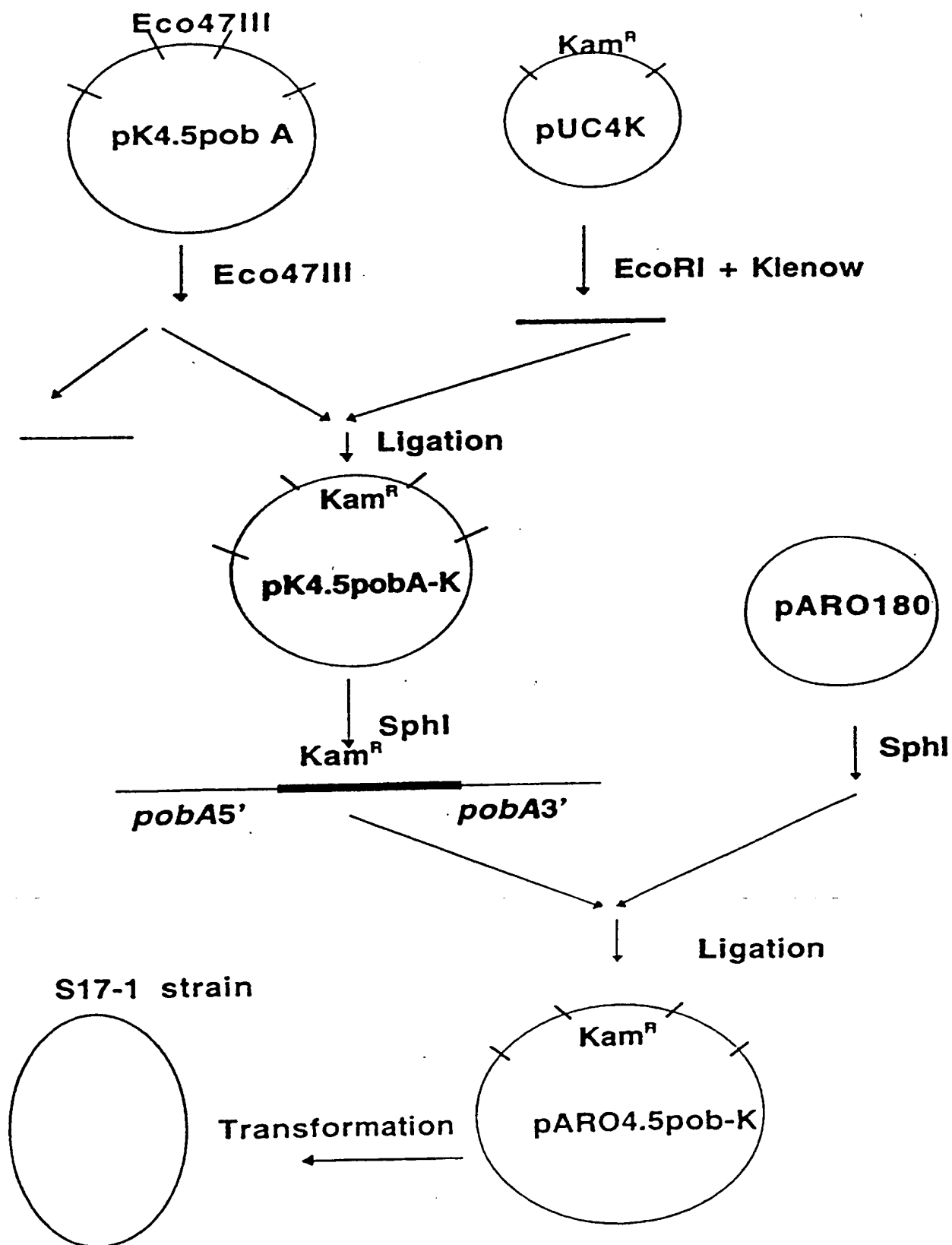
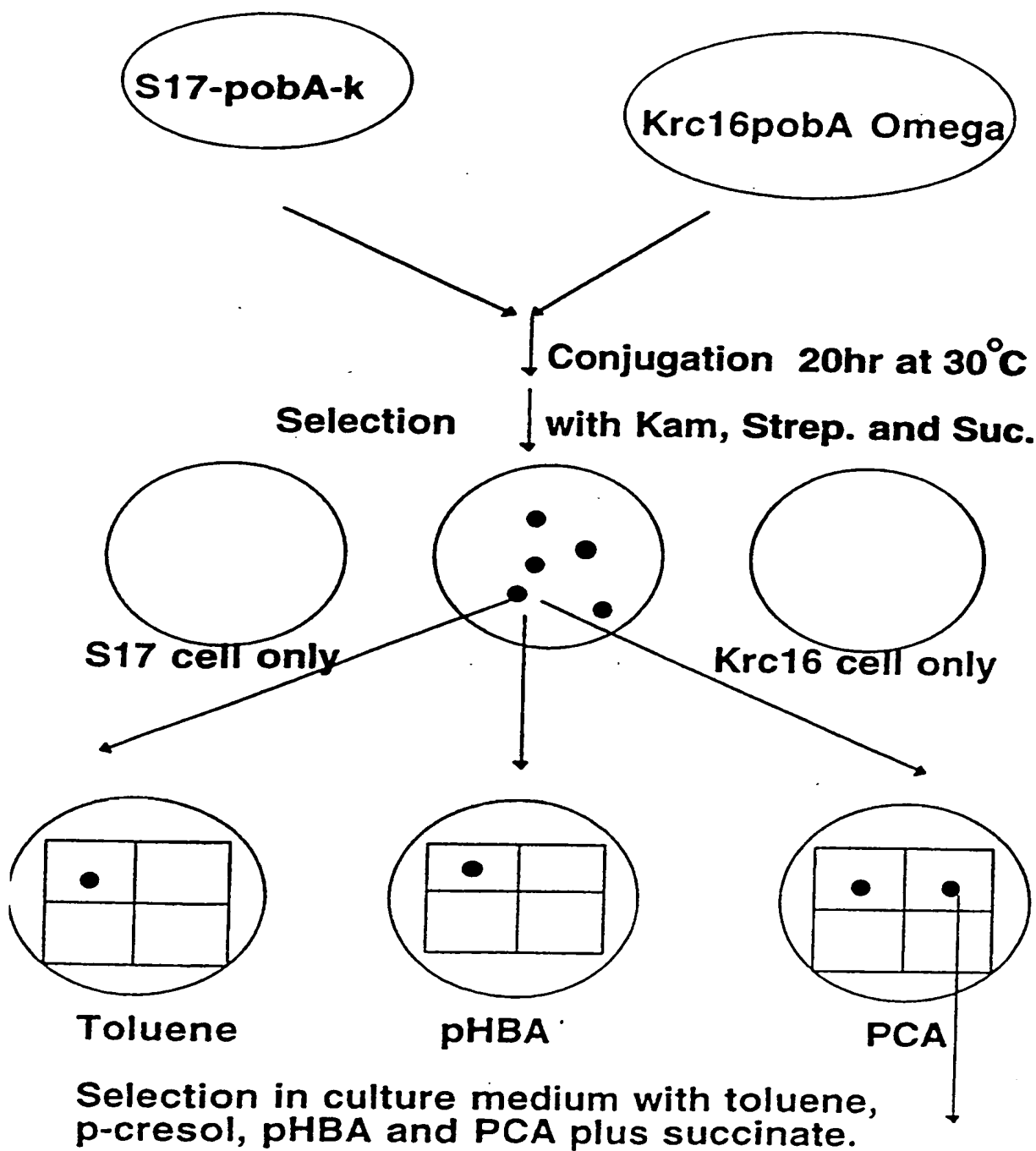


FIG.2



INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/12072

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ENTSCH, BARRIE ET AL: "Sequence and organization of pobA, the gene coding for p-hydroxybenzoate hydroxylase, an inducible enzyme from Pseudomonas aeruginosa" GENE (1988), 71(2), 279-91 CODEN: GENED6; ISSN: 0378-1119, XP002080557 cited in the application see the whole document ---	1
A	BARRY SHUMAN ET AL.: "Cloning, nucleotide sequence, and expression of a p-hydroxybenzoate hydroxylase isozyme gene from Pseudomonas fluorescens." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 268, no. 23, 15 August 1993, page 17057-17062 XP002080558 MD US cited in the application see the whole document ---	1
A	WO 92 06208 A (AMGEN INC) 16 April 1992 see claims -----	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

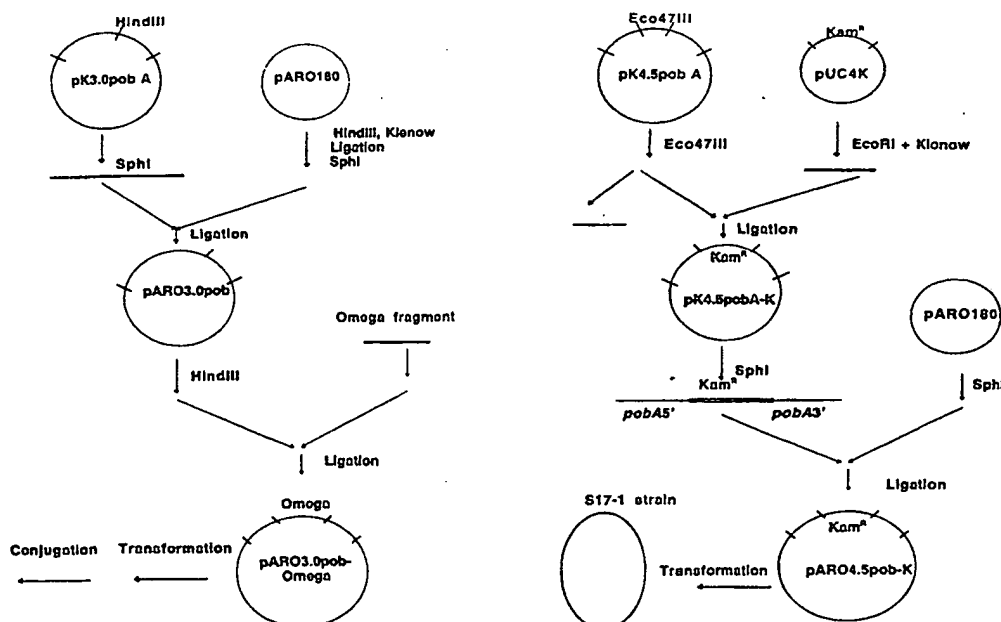
PCT/US 98/12072

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9206208 A	16-04-1992	US 5171684 A	15-12-1992
		AU 651142 B	14-07-1994
		AU 8651391 A	28-04-1992
		EP 0504349 A	23-09-1992
		JP 5502593 T	13-05-1993
		US 5605823 A	25-02-1997
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/52, 15/53, 9/02, C12P 7/42, C12N 1/21 // (C12N 1/21, C12R 1:38)		A1	(11) International Publication Number: WO 98/56920
			(43) International Publication Date: 17 December 1998 (17.12.98)
(21) International Application Number: PCT/US98/12072		(81) Designated States: AU, CA, IL, JP, NZ, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(22) International Filing Date: 11 June 1998 (11.06.98)			
(30) Priority Data: 60/049,556 13 June 1997 (13.06.97) US 09/090,044 3 June 1998 (03.06.98) US		Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.	
(71) Applicant (for all designated States except US): E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US).			
(72) Inventors; and (75) Inventors/Applicants (for US only): GRELAK, Rebecca, Lynn [US/US]; 784 Naamans Creek Road, Chadds Ford, PA 19317 (US). CHEN, Kevin, Kequin [CN/CN]; Novo Nordisk China, 22 Xinxu Zhong Lu, Shangdi Zone, Haidian District, Beijing 100085 (CN).			
(74) Agent: FLOYD, Linda, A.; E.I. du Pont de Nemours and Company, Legal Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).			

(54) Title: METHOD FOR THE PRODUCTION OF PARA-HYDROXYBENZOATE IN *PSEUDOMONAS MENDOCINA*

(57) Abstract

A transformed mutant, *P. mendocina*, is provided containing a DNA fragment that inactivates all *pobA* genes and encodes the enzyme hydroxybenzoate hydroxylase. Mutants deficient in hydroxybenzoate hydroxylase are useful for the production of para-hydroxybenzoate (PHBA).

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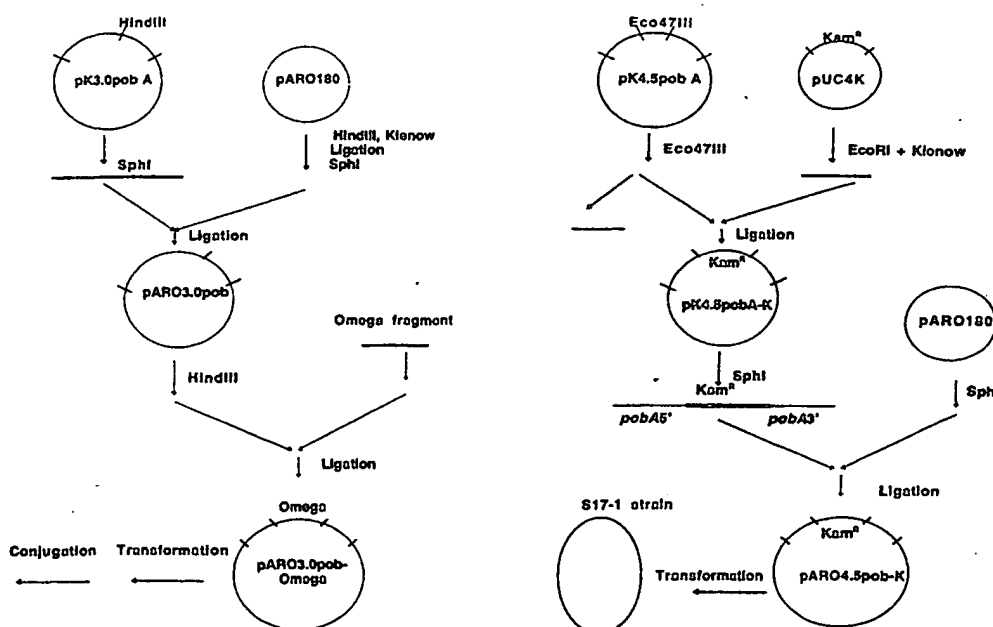
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/52, 15/53, 9/02, C12P 7/42, C12N 1/21 // (C12N 1/21, C12R 1:38)		A1	(11) International Publication Number: WO 98/56920
(21) International Application Number: PCT/US98/12072		(43) International Publication Date: 17 December 1998 (17.12.98)	
(22) International Filing Date: 11 June 1998 (11.06.98)		(81) Designated States: AU, CA, IL, JP, NZ, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(30) Priority Data: 60/049,556 13 June 1997 (13.06.97) US 09/090,044 3 June 1998 (03.06.98) US		Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.	
(71) Applicant (for all designated States except US): E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US).			
(72) Inventors; and			
(75) Inventors/Applicants (for US only): GRELAK, Rebecca, Lynn [US/US]; 784 Naamans Creek Road, Chadds Ford, PA 19317 (US). CHEN, Kevin, Kequin [CN/CN]; Novo Nordisk China, 22 Xinxu Zhong Lu, Shangdi Zone, Haidian District, Beijing 100085 (CN).			
(74) Agent: FLOYD, Linda, A.; E.I. du Pont de Nemours and Company, Legal Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).			

(54) Title: METHOD FOR THE PRODUCTION OF *para*-HYDROXYBENZOATE IN *PSEUDOMONAS MENDOCINA*

(57) Abstract

A transformed mutant, *P. mendocina*, is provided containing a DNA fragment that inactivates all *pobA* genes and encodes the enzyme hydroxybenzoate hydroxylase. Mutants deficient in hydroxybenzoate hydroxylase are useful for the production of *para*-hydroxybenzoate (PHBA).

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TITLEMETHOD FOR THE PRODUCTION OF *para*-HYDROXYBENZOATE
IN *PSEUDOMONAS MENDOCINA*FIELD OF INVENTION

5 The present invention relates to the fields of molecular biology and microbiology and the use of genetic techniques to modify existing enzymatic pathways for the production of desired compounds. More specifically, the present invention relates to a method for the production of *para*-hydroxybenzoate (PHBA) by a toluene-metabolizing *Pseudomonas mendocina* mutant lacking the
10 ability to express *para*-hydroxybenzoate hydroxylase (PHBH).

BACKGROUND

Para-hydroxybenzoate (PHBA) is the key monomer for Liquid Crystal Polymers (LCPs) which contain approximately 67% PHBA. Esters of PHBA also can be used as backbone modifiers in other condensation polymers, i.e.,
15 polyesters, and are also used to make parabens preservatives.

 Chemical synthesis of PHBA is known. For example, JP 05009154 teaches a chemical route using the Kolbe-Schmidt process from tar acid and CO₂ involving the extraction of tar acid from a tar naphthalene oil by an aqueous potassium hydroxide; adding phenol to the extracted tar acid potassium salt;
20 removing H₂O and reacting the resultant slurry with CO₂. Alternative methods of synthesis are also known; see, for example, US 5,399,178; US 4,740,614; and US 3,985,797.

 Chemical synthesis is problematic and costly due to the high energy needed for synthesis and extensive purification of product required. An alternate
25 low cost method with simplified purification would represent an advance in the art. Biological production offers one such low cost, simplified solution to this problem.

 Microbiological methods of PHBA synthesis are known. For example, JP 06078780 teaches PHBA preparation by culturing benzoic acid in the presence
30 of microorganisms (preferably *Aspergillus*) that oxidize benzoic acid to PHBA.

 An alternate method of biological production is suggested by bacteria that have an enzymatic pathway for the degradation of toluene and other organics where PHBA is produced as an intermediate. The first enzyme in the toluene degradation pathway is toluene monooxygenase (TMO) and the pathway is
35 referred to as the TMO pathway. Bacteria that possess the TMO pathway are primarily restricted to the genus *Pseudomonas* where *P. putida*, *P. fluorescens*, *P. aeruginosa* and *P. mendocina* are the most commonly utilized species. The TMO pathway has been highly characterized [Romine et al., Bioremediation of Chlorinated Polycyclic Aromatic Hydrocarbon Compounds (1994), 271-6.

Editor(s): Hinchee, Robert E. Publisher: Lewis, Boca Raton, FL] and a number of the genes encoding key enzymes have been cloned and sequenced, including the protocatechuate 3,4-dioxygenase genes [Frazee, *J. Bacteriol.*, (1993), 175(19), 6194-202], the *pcaR* regulatory gene from *Pseudomonas putida*, which is required
5 for the complete degradation of *p*-hydroxybenzoate [Romero-Steiner et al., *J. Bacteriol.* (1994), 176(18), 5771-9; Dimarco et al., *J. Bacteriol.* (1994), 176(14), 4277-84] and the *pobA* gene encoding the expression of *para*-hydroxybenzoate hydroxylase (PHBH), the principal enzyme for the conversion of PHBA to protocatechuate [Wong et al., *Microbiology* (Reading U.K.) (1994),
10 140(10), 2775-86; Entsch et al., *Gene* (1988), 71(2), 279-91].

Bacteria that possess the TMO pathway are useful for the degradation of toluene and trichloroethylene and are able to use these and other organics as sole carbon sources where they are transformed through PHBA to ring opening degradation products (US 5,017,495; US 5,079,166; US 4,910,143).

15 Recently, various strains of *Pseudomonas* possessing the TMO pathway have been used to produce muconic acid via manipulation of growth conditions (US 4,657,863; US 4,968,612). Additionally, strains of *Enterobacter* with the ability to convert *p*-cresol to PHBA have been isolated from soil (JP 05328981). Further, JP 05336980 and JP 05336979 disclose isolated strains of *Pseudomonas*
20 *putida* with the ability to produce PHBA from *p*-cresol.

Although the above cited methods are useful for the production of PHBA, these methods are limited by the high cost and toxicity of the aromatic substrate, *p*-cresol. Furthermore, the above methods use an isolated wildtype organism that converts part of the *p*-cresol to PHBA while the rest is further metabolized. The
25 utility of these methods are therefore limited by low yields and an inability to control further degradation of the desired product.

Shuman et al., (*J. Biol. Chem.*, (1993), 268, 17057) have reported the identification of a gene encoding a putative isozyme of PHBH from *P. fluorescens*. The comparison of the deduced amino acid sequence from this
30 gene with the enzyme encoded by the more fully characterized *pobA* gene of *P. fluorescens* (van Berkel et al., *Eur. J. Biochem.*; (1992), 210 (2), 411-419) demonstrates 73% homology with the isozyme. Although the work of Shuman et al. (supra) suggest the presence of a second gene encoding an alternate PHBH, the authors were not able to isolate any expressed protein and concluded that the gene
35 encoding the isozyme is not expressed.

Therefore, the problem to be overcome is to develop a method of microbially mediated PHBA production from an inexpensive, aromatic substrate, where the microbial TMO pathway has been altered to prevent the degradation of PHBA.

SUMMARY OF THE INVENTION

The present invention provides a method for the production of PHBA comprising: (i) culturing a *pobA(-)* *Pseudomonas* strain in a medium containing an aromatic organic substrate, at least one suitable carbon source, and a nitrogen source, wherein the *pobA(-)* *Pseudomonas* strain comprises genes encoding the TMO toluene degradation pathway and wherein the *pobA(-)* *Pseudomonas* strain does not produce any detectable *para*-hydroxybenzoate hydroxylase, whereby PHBA accumulates; and (ii) recovering the PHBA.

The invention further provides genes encoding novel *para*-hydroxybenzoate hydroxylase enzymes and *pobA(-)* *Pseudomonas* strains deficient in hydroxybenzoate hydroxylase that are able to accumulate PHBA under suitable growth conditions and in the presence of a suitable aromatic substrate.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1(a) schematically represents plasmid vectors used for the disruption of *pobA-1* alone in the variant of *P. mendocina*.

Figure 1(b) schematically represents plasmid vectors used for the disruption of *pobA-1* and *pobA-2* in the variant of *P. mendocina*.

Figure 2 is schematically represents the plasmid constructs needed for inactivation of *pobA-1* and *pobA-2*.

Figure 3 graphically compares the accumulation of PHBA in the *pobA(-)* *P. mendocina* mutants.

Figure 4 schematically shows the TMO enzymatic pathway.

Figure 5 is a GC/MS analysis of PHBA production (PHBA peak indicated) from *P. mendocina* strains containing disrupted *pobA-1* and *pobA-2* genes.

BRIEF DESCRIPTION OF BIOLOGICAL DEPOSITS

AND SEQUENCE LISTING

The transformed *P. mendocina* KRC1651 containing the omega-disrupted *pobA-1* and *pobA-2* genes was deposited on November 26, 1996, with the ATCC under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and is designated as ATCC 55885.

The fast-growing *P. mendocina* KRC16 was deposited on November 26, 1996 with the ATCC under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and is designated as ATCC 55886.

"ATCC" refers to the American Type Culture Collection international depository located at 10801 University Boulevard, Manassas, VA 20110-2209, U.S.A. The designations refer to the accession number of the deposited material.

Applicants have provided 10 sequences in conformity with Rules for the Standard Representation of Nucleotide and Amino Acid Sequences in Patent Applications (Annexes I and II to the Decision of the President of the EPO, published in Supplement No. 2 to OJ EPO, 12/1992) and with

5 37 C.F.R. 1.821-1.825 and Appendices A and B (Requirements for Application Disclosures Containing Nucleotides and/or Amino Acid Sequences).

SEQ ID NO: 1 gives the nucleotide sequence of the *pobA-1* gene isolated from *P. mendocina* KR-1.

10 SEQ ID NO: 2 gives the nucleotide sequence of the *pobA-2* gene isolated from *P. mendocina* KR-1.

SEQ ID NO: 3 gives the amino acid sequence of the *pobA-1* gene isolated from *P. mendocina* KR-1.

SEQ ID NO: 4 gives the amino acid sequence of the *pobA-2* gene isolated from *P. mendocina* KR-1.

15 SEQ ID NO: 5 gives the nucleotide sequence of the *pobA* gene isolated from *P. fluorescens* (van Berkel et al., *supra*.)

SEQ ID NO: 6 gives the nucleotide sequence of the *pobB* gene encoding the PHBH isozyme isolated from *P. fluorescens* (Shuman et al., *supra*.)

20 SEQ ID NO: 7 gives the amino acid sequence of the *pobA* gene isolated from *P. fluorescens* (van Berkel et al., *supra*.)

SEQ ID NO: 8 gives the amino acid sequence of the *pobB* gene encoding the PHBH isozyme isolated from *P. fluorescens* (Shuman et al., *supra*.)

25 SEQ ID NO:9 gives the nucleotide sequence of the 5' primer used to generate the *pobA* hybridization probe for the isolation of the *pobA-1* and *pobA-2* genes.

SEQ ID NO:10 gives the nucleotide sequence of the 3' primer used to generate the *pobA* hybridization probe for the isolation of the *pobA-1* and *pobA-2* genes.

DETAILED DESCRIPTION OF THE INVENTION

30 Applicants have developed a fast-growing *P. mendocina* mutant which lacks the PHBH enzyme and is unable to convert PHBA to PCA. The mutant metabolizes toluene and *p*-cresol resulting in the accumulation of PHBA in the growth medium. Applicants have made the unexpected finding that *P. mendocina* possesses two, highly homologous *pobA* genes, both of which
35 encode enzymes able to convert PHBA to PCA.

Applicants have also provided a method for the biological production of PHBA from genetically altered *Pseudomonas*. The method provides PHBA without the high energy cost of synthetic production and without producing toxic

waste streams. PHBA is a valuable monomer for the synthesis of liquid crystalline polymers.

The following definitions are to be used to interpret the terms contained in the claims and specification.

- 5 The terms "plasmid" and "vector" refer to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA molecules. Such elements may be autonomously replicating sequences, genome-integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded
- 10 DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell.
- 15 "Transformation vector" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that facilitate transformation of a particular host cell.

A "fragment" constitutes a fraction of the DNA sequence of the particular region.

- 20 The term "transformation" refers to the acquisition of new genes in a cell after the incorporation of nucleic acid.

- 25 The term "expression" refers to the transcription and translation to gene product from a gene coding for the sequence of the gene product. In the expression, a DNA chain coding for the sequence of gene product is first transcribed to a complimentary RNA which is often a messenger RNA and, then, the transcribed messenger RNA is translated into the above-mentioned gene product if the gene product is a protein.

The terms "restriction endonuclease" and "restriction enzyme" refer to an enzyme which binds and cuts within a specific nucleotide sequence within double-stranded DNA.

- 30 "Polymerase Chain Reaction" and "PCR" refer to a method that results in the linear or logarithmic amplification of nucleic acid molecules. PCR generally requires a replication composition consisting of, for example, nucleotide triphosphates, two primers with appropriate sequences, DNA or RNA polymerase and proteins. These reagents and details describing procedures for their use in
- 35 amplifying nucleic acids are provided in U.S. 4,683,202 (1987, Mullis et al.) and U.S. 4,683,195 (1986, Mullis et al.).

"Gene" refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding) and following (3' non-coding) the coding region. "Native" gene refers to an isolated gene with its own

regulatory sequences as found in nature. "Chimeric gene" refers to a gene that comprises heterogeneous regulatory and coding sequences not found so assembled in nature. "Endogenous" gene refers to the native gene normally found in its natural location in the genome and is not isolated.

5 "TMO" is the abbreviation for toluene monooxygenase-1.

The terms "TMO degradative pathway" or "TMO enzymatic pathway" refer to the enzymes and genes encoding the enzymes found in some *Pseudomonas* bacteria that are responsible for the degradation of toluene and similar aromatic substrates. The TMO pathway is outlined in Figure 4 and
10 contains at least toluene-4-monooxygenase, *p*-cresol methylhydroxylase, *p*-hydroxybenzoaldehyde dehydrogenase, and *p*-hydroxybenzoate hydroxylase.

"PHBH" is the abbreviation for *para*-hydroxybenzoate hydroxylase, the enzyme in the TMO pathway responsible for the conversion of PHBA to PCA.

"PHBA" is the abbreviation for *para*-hydroxybenzoate which is also
15 known as *para*-hydroxybenzoic acid or 4-hydroxybenzoic acid.

"PCA" is the abbreviation for protocatechuic acid.

"EDTA" is the abbreviation for ethylenediaminetetraacetic acid.

The term "*pobA*" refers to a gene encoding the *para*-hydroxybenzoate hydroxylase enzyme.

20 The term "*pobB*" refers to the gene encoding the putative isozyme of PHBH from *P. fluorescens*.

"*pobA-1*" refers to a first gene encoding *para*-hydroxybenzoate hydroxylase and having the sequence as given by SEQ ID NO:1 and is contained within a 3.0 kb fragment of *P. mendocina* genomic DNA isolated by hybridization
25 using a *pobA* hybridization probe. "*pobA-2*" refers to a second gene encoding *para*-hydroxybenzoate hydroxylase and having the sequence as given by SEQ ID NO:2 and is contained within a 4.5 kb fragment of *P. mendocina* genomic DNA isolated by hybridization using a *pobA* hybridization probe.

"*pobR*" refers to a transcriptional activator gene required for the
30 expression of *pobA* genes in *Pseudomonas* sp.

The term "*pobA* hybridization probe" refers to a 1.2 kb PCR amplification product produced by amplification of *P. mendocina* genomic DNA with primers based on the nucleotide sequence of the *P. fluorescens pobA* gene [SEQ ID NO: 5] as described by van Berkel et al., *Eur. J. Biochem.* (1992), 210 (2), 411-419.

35 The term "*pobA*(-) *Pseudomonas*" refers to a genetically manipulated *Pseudomonas* having mutations or disruptions in all *pobA* genes such that the genetically manipulated *Pseudomonas* is unable to produce active *para*-hydroxybenzoate hydroxylase.

The term "aromatic organic substrate" refers to an aromatic compound that is degraded by the TMO enzymatic pathway. Typical examples of suitable aromatic substrates are toluene and *p*-cresol.

5 The term "carbon source" refers to a substrate suitable for bacterial cell growth that is distinct from the aromatic substrate. Suitable carbon substrates include but are not limited to glucose and succinate.

10 The term "TMO *P. mendocina* variant" refers to a genetic variant of a wildtype *P. mendocina* capable of metabolizing toluene and demonstrating an increase in cell density of at least 6 fold greater than the wildtype under growth conditions of approximately 160 ppm toluene as a sole carbon source. Variants are typically produced by standard mutagenic methods and screened for higher growth rate and the ability to metabolize toluene and other aromatic substrates.

15 The term "suicide vector" refers to a vector generally containing a foreign DNA fragment to be expressed in a suitable host cell, coupled with a genetic element that will be lethal to the host cell unless the cell is able to express the foreign DNA. "Suicide vector" is also understood to mean a non-replicating vector capable of transfecting a host cell and facilitating the incorporation of foreign DNA into the genome of the host cell. Such a vector does not replicate and is thus destroyed after incorporation of the heterologous DNA. Examples of
20 common suicide vectors and their construction may be found in (Sambrook et al., Molecular Cloning: A Laboratory Manual - volumes 1,2,3 (Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1989)).

The term "*Kam*" refers to a gene encoding Kanamycin resistance.

The term "*Amp*" refers to a gene encoding Ampicillin resistance.

25 The term "*Strep*" refers to a gene encoding Streptomycin resistance.

Applicants' method involves mutagenesis of a toluene-metabolizing *P. mendocina* strain such that the mutant strain demonstrates a cell density of at least 6 fold greater than the wildtype under growth conditions of approximately 160 ppm toluene. The fast-growing TMO variant was then genetically altered so
30 that all genes encoding *para*-hydroxybenzoate hydroxylase (PHBH) were disrupted. *P. mendocina* strains containing the disrupted genes (*pobA-1*, *pobA-2*), referred to as *pobA*(-) mutants, were then grown in the presence of toluene or *p*-cresol and PHBA was accumulated in the medium.

35 Creation of *pobA*(-) mutants proceeded by (i) the isolation of a *pobA* hybridization probe; (ii) isolation of *pobA-1* from a TMO variant using the probe; (iii) disruption of *pobA-1* using an omega element and subsequent cloning and expression into a suicide vector which confirmed the existence of an alternate pathway for PHBA catabolism; (iv) isolation of *pobA-2*; (v) disruption of both *pobA-1* and *pobA-2*; and (vi) cloning into a suicide vector for the creation of a

pobA(-) mutant TMO variant that accumulates PHBA in the presence of toluene or *p*-cresol and an alternate carbon source.

One of the most significant aspects in the development of the present method is the discovery that the instant strains contained two expressed *pobA* genes, both of which needed to be inactivated before PHBA accumulation could be effected. Another significant aspect of the invention is the creation of a toluene-metabolizing *P. mendocina* strain that has a significantly higher rate of growth and metabolism than many toluene-metabolizing strains known in the art. The fast-growing TMO variant provides a host useful for the commercial production of PHBA and that permits rapid screening of *pobA(-)* transformants.

PRODUCTION CONDITIONS

TMO-Containing Bacterial Strains

Bacterial cells preferred in the present invention are those that possess the TMO pathway. Such strains are generally restricted to the genus *Pseudomonas* and include but are not limited to *P. putida*, *P. fluorescens*, *P. aeruginosa* and *P. mendocina*.

Strains of *Pseudomonas* containing the TMO pathway are known to oxidize toluene to form intermediates of the tricarboxylic acid cycle. PHBA as well as other intermediates, such as *p*-cresol, *p*-hydroxybenzyl alcohol and *p*-hydroxybenzaldehyde, are formed in the upper pathway, which metabolizes toluene to the ring cleavage substrate (Figure 4). In wildtype *Pseudomonas* strains, PHBA is immediately converted to protocatechuate (PCA) as it is formed. The biochemistry of the enzymes involved in the upper pathway have been described for several *Pseudomonas* strains (Romine et al., *supra*).

Batch and Continuous Fermentations

The present process uses a batch method of fermentation. A classical batch fermentation is a closed system where the composition of the medium is set at the beginning of the fermentation and not subjected to artificial alterations during the fermentation. Thus, at the beginning of the fermentation the medium is inoculated with the desired organism or organisms and fermentation is permitted to occur adding nothing to the system. Typically, however, a batch fermentation is "batch" with respect to the addition of carbon source and attempts are often made at controlling factors such as pH and oxygen concentration. In batch systems the metabolite and biomass compositions of the system change constantly up to the time the fermentation is stopped. Within batch cultures, cells moderate through a static lag phase to a high growth log phase and finally to a stationary phase where growth rate is diminished or halted. If untreated, cells in the stationary phase will eventually die.

A variation on the standard batch system is the Fed-Batch system.

Fed-Batch fermentation processes are also suitable in the present invention and comprise a typical batch system with the exception that the substrate is added in increments as the fermentation progresses. Fed-Batch systems are useful when catabolite repression is apt to inhibit the metabolism of the cells and where it is desirable to have limited amounts of substrate in the medium. An advantage of the Fed-Batch system is that it is more amenable to the use of toxic or immiscible aromatic substrates such as toluene. Using a Fed-Batch system it is possible to maintain a steady concentration of substrate at non-toxic levels while accommodating maximum bioconversion of the substrate to product.

The production of PHBA from aromatic compounds such as toluene will be limited by the amount of the aromatic substrate and carbon sources added. In simple Batch fermentation, production will be limited by the amount of toluene initially added. Since toluene is toxic and has limited solubility in water, its low initial concentration will govern the amount of PHBA produced. The ability to run the process at such a low toluene (i.e., 30-60 ppm) allows operation below a lower explosive limit which for toluene is 120 ppm. This is a clear safety advantage to the process. Fed-Batch techniques where the carbon source and toluene are added at rates which are similar to the utilization of these compounds will keep the toluene concentration in the medium low and can significantly increase the amount of PHBA produced.

Batch and Fed-Batch fermentations are common and well known in the art and examples may be found in, for example Thomas D. Brock in *Biotechnology: A Textbook of Industrial Microbiology*, Second Edition (1989) Sinauer Associates, Inc., Sunderland, MA., or Deshpande, Mukund V. *Appl. Biochem. Biotechnol.* 36, 227, (1992).

Although the present invention is performed in batch mode, it is contemplated that the method would be adaptable to continuous fermentation methods. Continuous fermentation is an open system where a defined fermentation medium is added continuously to a bioreactor and an equal amount of conditioned medium is removed simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density where cells are primarily in log phase growth.

Continuous fermentation allows for the modulation of one factor or any number of factors that affect cell growth or end product concentration. For example, one method will maintain a limiting nutrient such as the carbon source or nitrogen source at low concentration and allow all other parameters to be in excess. In other systems a number of factors affecting growth can be altered continuously while the cell concentration, measured by medium turbidity, is kept

constant. Continuous systems strive to maintain steady state growth conditions and thus the cell loss due to medium being drawn off must be balanced against the cell growth rate in the fermentation. Methods of modulating nutrients and growth factors for continuous fermentation processes as well as techniques for
5 maximizing the rate of product formation are well known in the art of industrial microbiology and a variety of methods are detailed by Brock, *supra*.

It is contemplated that the present invention may be practiced using either batch, Fed-Batch or continuous processes and that any known mode of fermentation would be suitable. Additionally, it is contemplated that cells may be
10 immobilized on a substrate as whole cell catalysts and subjected to fermentation conditions for PHBA production.

Fermentation Studies and Culture Conditions

The present invention provides a process for the production of PHBA in a culture medium by *P. mendocina* comprising culturing a genetically engineered
15 *P. mendocina* host having no *p*-hydroxybenzoate hydroxylase where all *pobA* genes are disrupted. The medium may optionally contain a nitrogen source, minor metals, a carbon source and an aromatic substrate. The culture medium can contain any inorganic or organic source of nitrogen normally used in processes for culturing microorganisms. Nitrogen sources may be either inorganic (ammonium salts, e.g., ammonium sulfate) or organic nitrogen (e.g., yeast extract).
20

Growth Conditions

Typically, cells are cultured in flasks and fermenters and the fermentation modes used were batch, Fed-Batch and continuous. Both "rich" and "lean" media were used as described in the GENERAL METHODS. Culture conditions were
25 modulated according to the method of growth and optimized for the production of PHBA. The pH of the cultures should be maintained within a range of about from 6.3 to 7.9 wherein a range from about 7.2 to 7.7 is most preferred. Methods for the optimization of cell growth and metabolism are well known in the art, for example as described in Manual of Methods for General Bacteriology (Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A.
30 Wood, Noel R. Krieg and G. Briggs Phillips, eds), American Society for Microbiology, Washington, DC. (1994).

Carbon Source

A variety of carbon sources are suitable in the present invention and
35 include but are not limited to materials such as succinate, lactate, acetate, ethanol, monosaccharides such as glucose and fructose, oligosaccharides such as lactose or sucrose, polysaccharides such as starch or cellulose or mixtures thereof and unpurified mixtures from renewable feedstocks such as cheese whey permeate, cornsteep liquor, sugar beet molasses, and barley malt. The choice of the carbon

substrate will be determined by the needs of the desired production cell. For the purposes of the present invention, glucose is preferred.

In *pobA(-)* *P. mendocina* cultures, a carbon source is necessary as the TMO pathway is completely blocked, and the strain is not able to utilize the aromatic substrate as a carbon source for the production of energy. In practice, it is necessary to balance the concentration of the carbon substrate against the concentration of the aromatic substrate in order to control the amount of oxidation of the aromatic substrate at the beginning of the TMO pathway.

Aromatic substrates

A variety of aromatic substrates may be used in the present invention, including but not limited to toluene, *p*-cresol, *p*-hydroxybenzyl alcohol, *p*-hydroxybenzaldehyde, benzoate and any aromatic compounds where the chemical structure is similar to toluene and the intermediates of the TMO pathway.

The production of PHBA from aromatic substrates such as toluene and *p*-cresol will be limited by the concentration of the substrate and the carbon source in the medium. Preferred concentrations of toluene are from about 30 ppm to about 500 ppm where a range of about 30 ppm to about 60 ppm is most preferred and a range of about 30 ppm to 60 ppm is most preferred. Preferred concentrations of *p*-cresol are from about 1 mM to about 5 mM.

CLONING AND SEQUENCING OF *pobA-1* GENE

Development of fast-growing TMO *P. mendocina* variant

The present invention provides a fast-growing *Pseudomonas* strain that exhibits a cell density of at least 6 fold greater than the wildtype under growth conditions of 0.2% toluene. The fast-growing strain is referred to as a TMO variant. Preferred *Pseudomonas* strains will be those that are able to metabolize toluene and possess the key enzymes in the TMO enzymatic pathway (Figure 4). Strains known to possess this pathway and useful in the present invention include but are not limited to *P. putida*, *P. fluorescens*, *P. aeruginosa* and *P. mendocina*. Most preferred are the *P. mendocina* strains KRC16 (ATCC 55886) and KRC50.

The TMO variant was developed by U.V. mutagenesis of a *P. mendocina* strain (KR-1) (Amgen, U.S. 5,171,684) known to be able to use toluene as a sole carbon source. After mutagenesis, cells were plated on a standard medium containing toluene and colonies were picked on the basis of size and speed of development. In this fashion KRC50 was isolated. A second mutation of KRC50 produced KRC16 (ATCC 55886) which exhibited a cell density of at least 6 fold higher than the parent KR-1 under growth conditions of approximately 160 ppm toluene. KRC16 was then used for further genetic studies.

Methods of mutagenesis useful in the invention are not limited to the use of U.V. light protocols but encompass a variety of agents such as chemical agents including HNO_2 and NH_2OH , as well as compounds that affect replicating DNA such as acridine dyes, notable for causing frameshift mutations. Specific methods for creating mutants using radiation or chemical agents are well documented in the art. See, for example, Brock, *supra*.

Isolation of *pobA-1* with a *pobA* hybridization probe

Identification of the *pobA-1* gene in the TMO variant KRC16 (ATCC 55886) proceeded by first amplifying genomic DNA from KRC16 with primers designed from the known base sequence of *pobA* isolated from *P. fluorescens* (van Berkel et al., *supra*). PCR amplification (U.S. 4,683,202 (1987, Mullis et al.) and U.S. 4,683,195 (1986, Mullis et al.) produced a 1.2 kb fragment that was used to probe KRC16 genomic DNA. Analysis of the genomic DNA by Southern blot (Southern, *J. Mol. Biol.* 98, 503, (1975)) revealed two bands by gel electrophoresis. One band, migrated at about 3.0 kb and the other migrated at 4.5 kb. The 3.0 kb band was excised and used for cloning and sequencing. Sequence analysis revealed the base sequence of *pobA-1* as given in SEQ ID NO:1. Comparison of the deduced amino acid sequence from the base sequence of *pobA-1* [SEQ ID NO:3] with the known peptide encoded by the *pobA* gene of *P. fluorescens* [SEQ ID NO:7] (van Berkle et al., *supra*) show 68% similarity suggesting *pobA-1* encodes *P. mendocina* PHBH.

Cloning *pobA-1* gene and confirmation of expression

The 3.0 kb fragment was re-isolated from agarose gel and cloned into an appropriate vector for transformation into *E. coli* in order to confirm *pobA-1* expression of active PHBH. Vectors suitable for the transformation of *E. coli* are numerous and well known in the art. Suitable vectors can be derived, for example, from a bacterium, a virus (such as bacteriophage T7 or a M-13 derived phage), a cosmid, a yeast or a plant. Protocols for obtaining and using such vectors are known to those in the art. (Sambrook et al. *Molecular Cloning: A Laboratory Manual* - volumes 1,2,3 (Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1989)).

PobA-1 was cloned into a *sphI* site of a pUC18 vector (Parke, D., *Gene* (1990), 93(1), 135-7), creating pUC3.0K which was used to transform the *E. coli* strain DH5 α . Transformants were screened for inserts containing the 3.0 kb fragment. Positive transformants were then grown in the presence of PHBA and toluidine to test for the expression of active PHBH by *pobA-1*. Conversion of PHBA to PCA was indicated by a colored reaction product formed by the reaction of toluidine and PCA. This method of assay is common and well known in the art; see, for example, "Application of *p*-Toluidine in Chromogenic Detection of

Catechol and Protocatechuate, Diphenolic Intermediates in Catabolism of Aromatic Compounds" (Parke, *Applied and Environmental Microbiology*, Aug. 1992, P.2694-2697).

DISRUPTION OF *pobA-1*

5 Cloning of the 3.0 kb *pobA-1* gene fragment into suicide vector

In order to clone the *pobA-1* gene into a suicide vector amenable to manipulation for gene disruption, the 3.0 kb fragment was re-isolated from pUC3.0k and cloned into the *sphI* site of the suicide vector, *pARO180* (Park, D., *supra*) to form the vector *pARO3.0pobA*. (Figure 1(a))

10 Construction of suicide vectors are common and well known in the art and have been developed to transfect a variety of microorganisms to facilitate genetic manipulations. For example, suicide vectors capable of transfecting *Salmonella* (Galen et al., 96th General Meeting of the American Society for Microbiology, New Orleans, Louisiana, USA, May 19-23, 1996. Abstracts of the General
15 Meeting of the American Society for Microbiology 96 (0), (1996) 529); *Streptococcus* (Brooker et al., *Letters in Applied Microbiology* 21 (5) (1995) 292-297); *Rhizobium* (Safronova et al., *Genetika* 30 (6) (1994) 763-768); *Rhodobacter* (Penfold et al., *Gene* (Amst) 118 (1) (1992) 145-146) and other
20 gram-negative bacteria (Skrzypek et al., *Plasmid* 29 (2) (1993) 160-163), have all been constructed. Suicide vectors will be constructed so as to accommodate the specific host to be transfected and to accomplish the desired purpose. Generally suicide vectors useful in the present invention are non-replicating and capable of
25 transfecting a host cell with foreign DNA and facilitating incorporation of that DNA into the genome of the host cell. Such vectors are destroyed after transfecting the desired host with foreign DNA.

Within the context of the present invention, a mobilizing, narrow host range vector, which will replicate to high copy number in *E. coli* is preferred. Such vectors will be able to be efficiently transferred to other gram-negative bacteria from a mobilizing host strain such as S17-1 (Parke et al., *supra*). For the
30 purposes of the present invention a variety of suicide vectors will serve to carry the *pobA* gene for transfection into a suitable host where *pARO180* is preferred.

Disruption of *pobA-1* gene with Omega element

In order to prevent expression of the cloned *pobA-1* gene, a nucleic acid disrupting fragment known as an omega element was cloned into a *HindIII* site
35 within the coding region of the *pobA-1* gene on the vector *pARO3.0pobA*. The omega element carries a gene for Streptomycin resistance and is fully described in Prentki et al., *Gene* 29, 303, (1984). The vector containing the disrupted *pobA-1* was then used to transform a mobilizing *E. coli* strain for eventual transfer into the *P. mendocina* KRC16 (ATCC 55886).

The process of gene disruption analysis is common and well known in the art. Gene disruption involves inserting a non-coding DNA fragment or a DNA fragment of a selectable marker in the coding region of a target structural gene and transforming a host containing an endogenous target gene with that disrupted construct. Homologous recombination between the disrupted gene and the endogenous gene will result in inactivation of the endogenous gene in the host organism. Gene disruption is used in a variety of organisms such as *Mycobacterium* (Balasubramanian et al., *Journal of Bacteriology* 178 (1) (1996) 273-279); *Saccharomyces* (Daran et al., *European Journal of Biochemistry* 233 (2) (1995) 520-530); *Brevibacterium* (Su Y-C et al., Proceedings of the National Science Council Republic of China Part B, Life Sciences 19 (2) (1995) 113-122); as well as *E. coli* (Cherepanov et al., *Gene* (Amsterdam) 158 (1) (1995) 9-14).

To transfer the plasmid containing the disrupted *pobA-1* gene to KRC16 (ATCC 55886), the transformed mobilizing *E. coli* was incubated in a 1:4 ratio with KRC16 and plated on a standard medium. Positive transformants were selected on the basis of antibiotic resistance and the presence of the disrupted *pobA-1* gene was confirmed by re-isolation of the gene by restriction analysis and hybridization with the 1.2 kb *pobA* hybridization probe. This process is called transformation by conjugation. Alternatively, cells may be transformed by known procedures such as by protocols involving calcium-permeabilized cells, electroporation, or by transfection using a recombinant phage virus. (Sambrook et al., *supra*)

The disrupted *pobA-1* gene is an integrating DNA fragment which has a DNA sequence with homology to a target site in the genome of the host cell so that the chimerical DNA fragment of *pobA-1* gene and a drug-resistance gene can be integrated into the genome of the host *P. mendocina*.

ANALYSIS OF PHBA CONVERSION WITH *pobA-1* DISRUPTED STRAINS

Transformed KRC16 (ATCC 55886) carrying the disrupted *pobA-1* gene was grown in the presence of toluene or *p*-cresol under suitable conditions for the production of PHBA. Analysis of the culture supernatant by GC/Mass spectrometry indicated that only marginal PHBA accumulation occurred while there was significant production of PCA. These data suggested that the disruption of *pobA-1* did not block the TMO pathway at the PHBH step and that the *P. mendocina* KRC16 (ATCC 55886) mutant carrying the disrupted *pobA-1* gene was still capable of converting PHBA to PCA.

Since the initial analysis of KRC16 genomic DNA had indicated a 4.5 kb fragment resulting from hybridization with the *pobA* probe, in addition to the 3.0 kb fragment, it was decided to investigate whether this 4.5 kb fragment

represented an alternate *pobA* gene capable of mediating the conversion of PHBA to PCA in the presence of a disrupted *pobA-1* gene.

CONSTRUCTION OF A *P. MENDOCINA* KRC16 CONTAINING TWO DISRUPTED *pobA* GENES

5 Isolation, disruption and cloning of *pobA-2*

The 4.5 kb fragment was isolated from a gel following restriction of *P. mendocina* genomic DNA and probing with the *pobA* hybridization probe, essentially in the same fashion as the isolation of the 3.0 kb fragment. The isolated gene was referred to as *pobA-2*. Sequence analysis revealed the base
10 sequence of *pobA-2* as given in SEQ ID NO:2. Comparison of the deduced amino acid sequence from the base sequence of *pobA-2* [SEQ ID NO:4] with the known peptide encoded by the *pobB* gene for a putative PHBH isozyme [SEQ ID NO:8] of *P. fluorescens* (Shuman et al., *supra*) show 71.2% similarity suggesting *pobA-2* encodes *P. mendocina* PHBH.

15 Disruption of *pobA-2*

In order to create a disrupted *pobA-2* gene the isolated *pobA-2* gene was cloned into a pUC4K vector (Pharmacia Biotech, Piscataway, NJ, 08854, USA) and then the gene was disrupted by the insertion of a *Kam* resistance gene cassette into an Eco47III site in the *pobA-2* gene. The resulting plasmid was termed
20 pK4.5pobA-Kam (Figure 1(b)). Digestion of pK4.5pobA-Kam with *sphI* and subsequent ligation with the suicide vector pARO180 produced pAR04.5pob-Kam. pAR04.5pob-Kam was moved into a mobilizing *E. coli* strain and KRC16, containing the omega-disrupted *pobA-1* gene was transformed. A number of transformants were selected and found to contain the disrupted *pobA-2*
25 gene. Eight strains (KRC1615, KRC1616, KRC1651 (ATCC 55885), KRC1617, KRC1645, KRC1652, KRC1653, and KRC1657) were selected for further study. The presence of both disrupted genes was confirmed by re-isolation of the genes by restriction analysis and hybridization with the 1.2 kb *pobA* hybridization probe.

30 PHBA ACCUMULATION IN KRC1615, KRC1616, KRC1651 (ATCC 55885), KRC1617, KRC1645, KRC1652, KRC1653, AND KRC1657

Eight *P. mendocina* strains, (KRC1615, KRC1616, KRC1651 (ATCC 55885), KRC1617, KRC1645, KRC1652, KRC1653, and KRC1657), containing disrupted *pobA-1* and *pobA-2* genes, were grown in the presence of toluene or *p*-cresol and an alternate carbon source and the culture supernatant was
35 analyzed for accumulation of PHBA and PCA. Analysis by GC/MS indicated that no PCA was formed. KRC1651 (ATCC 55885) appeared to be the highest producer of PHBA. A comparison of the relative PHBA production of all strains is seen in Figure 3.

METHODS OF PHBA ASSAY

PHBA may be detected by a variety of means including Gas chromatography coupled with Mass spectrophotometry (GC/MS) or methods using capillary electrophoresis and HPLC. Capillary electrophoresis is preferred for rapid analysis where a large number of samples are involved, whereas GC/MS is noted for its sensitivity, accuracy and the ability to confirm the correct chemical structure of the compound being characterized.

Methods for determining the existence of PHBA and toluene derivatives by GC/MS are known in the art (see, for example, Adachi et al., *Meijo Daigaku Nogakubu Gakujutsu Hokoku* (1996) 32, 53-57; Luczak et al., *Fitoterapia* (1991) 62(5), 455-6; Uda et al., *Nippon Shokuhin Kogyo Gakkaishi* (1988) 35(5), 360-6). The present methods are based on those of the literature and are more fully described in the GENERAL METHODS.

Methods for determining PHBA by capillary electrophoresis are also known in the art (see, for example, Chen et al., *Fenxi Huaxue* (1995) 23(11), 1281-3; Wu et al., *J. Chromatogr., A* (1995) 716(1 + 2), 291-301). Capillary electrophoresis may be used in the present invention to detect PHBA as well as the disappearance of toluene and *p*-cresol from the culture supernatant of a *pobA*(-) *P. mendocina* strain.

Capillary electrophoresis uses a combination of electrophoretic migration and electroosmotic flow to separate molecules based on differences in charge, size, isoelectric focusing and hydrophobicity.

REGULATION OF THE *pobR* REPRESSOR

Although the foregoing describes only the genetic manipulation of the *pobA* gene in the *P. mendocina* TMO pathway, it is contemplated that other regulatory genes may also be manipulated to effect PHBA and other TMO intermediate metabolite accumulation. One such regulatory gene is the *pobR* gene.

pobR is a transcriptional activator required for the expression of *pobA* gene (DiMarco et al., *J. Bacteriol.* (1993) 175(14), 4499-506; DiMarco et al., *J. Bacteriol.* (1994) 176(14), 4277-84). Applicants have isolated and sequenced two *pobR* genes from *P. mendocina* KRC16, which are located upstream of *pobA-1* and *pobA-2*. It is possible that each *pobA* gene in *P. mendocina* contains a region which may be involved in sequence-specific recognition by *pobR*, forming the core of the operator site as reported in *Acinetobacter calcoaceticus* (DiMarco, (1994), *supra*). In the presence of an inducer such as PHBA, association of *pobR* with its ligand fosters binding by RNA polymerase and leads to transcription of *pobA*. It is contemplated that an alternative approach to generating a PHBA-producing *P. mendocina* strain would involve the manipulation of the *pobR* genes

controlling each respective *pobA* gene. For example, *pobR* genes could be genetically altered or disrupted so as to prevent transcription of all *pobA* genes, resulting in inhibited PHBH expression and accumulation of PHBA in the altered cells.

- 5 The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit
10 and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

EXAMPLES

GENERAL METHODS

- 15 Procedures for the genetic manipulations of cellular genomes are well known in the art. Techniques suitable for use in the following examples may be found in Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989).

- 20 Materials and methods suitable for the maintenance and growth of bacterial cultures are well known in the art. Techniques suitable for use in the following examples may be found in Manual of Methods for General Bacteriology (Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, eds), American Society for Microbiology, Washington, DC. (1994) or Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates,
25 Inc., Sunderland, MA. All reagents and materials used for the growth and maintenance of bacterial cells were obtained from Aldrich Chemicals (Milwaukee, WI), DIFCO Laboratories (Detroit, MI), GIBCO/BRL (Gaithersburg, MD), or Sigma Chemical Company (St. Louis, MO) unless otherwise specified.

- 30 The meaning of abbreviations is as follows: "h" means hour(s), "min" means minute(s), "sec" means second(s), "d" means day(s), "mL" means milliliters, "L" means liters.

Cell strains:

 The *Pseudomonas* strains KRC16 (ATCC 55886) and KRC50 were used for transformation and production of PHBA.

35 Growth Conditions

 Typically, studies were conducted in flasks and 1&10-L fermenters and the fermentation modes used were batch, Fed-Batch and continuous. The medium most commonly used was a basal medium containing 0.48 g/L of yeast extract ("lean medium") or the same medium but with 4 g/L yeast extract and 10 g/L

N-Z-amine E ("rich medium" Sheffield Products, Kraft Inc.), glucose was the carbon source and ammonia the inorganic nitrogen source.

Rich medium is available commercially from a variety of sources as from GIBCO/BRL (Gaithersburg, MD) for example. Other media amenable to the procedures of the present invention are common in the art and are fully described in Manual of Methods for General Bacteriology (Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, eds), American Society for Microbiology, Washington, DC. (1994).

It is preferred that the cells are cultured in a 2-phase process, where the cells are first grown to a suitable cell density in rich medium in the first stage and then transferred to a production medium containing the aromatic substrate for PHBA production in a second stage. Preferred cell densities for the first and second stage culture are from about 0.1 g/L to about 50 g/L.

Analysis of Various Toluene Metabolites by Gas Chromatography

Compounds were prepared for GC analysis as follows: 10 mg of each compound was dissolved in 500 μ L of ethylacetate and 500 μ L of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA). This mixture was heated for 30 min at approximately 80 °C to allow derivatization of the compounds. The reaction was then allowed to cool down to room temperature and the volume was adjusted to 1.0 mL as needed. A 1:10 dilution was performed with all compounds and 100 μ L of each solution was dispersed into a GC vial. Samples were analyzed by injecting 1.0 μ L (1000 ng) into the column. The GC conditions used for analysis were: Initial temp. 100 °C for 5 min; increased to 200 °C over 20 min at 5 °C/min. The GC column used was HP-5 cross linked 5% Phenylmethyl siloxane (30 M x 0.32 ID). Using these conditions, the following retention times (min) were obtained for various compounds tested: *p*-cresol, 4.27; 4-hydroxybenzaldehyde, 10.22; 4-hydroxybenzylalcohol, 14.05; 4-hydroxybenzoic acid (PHBA), 17.12; protocatechuic acid (PCA), 21.74.

EXAMPLE 1

CLONING AND SEQUENCING OF *pobA-1* GENE

Mutagenesis of *P. mendocina* KR1

A colony of *P. mendocina* KR-1 (Amgen, Thousand Oaks, CA) was inoculated into about 3 mL LB medium and the culture shaken at 30 °C overnight. Cells were diluted by 100 to 500 fold with LB medium and plated on agar plates of LB medium. Plates were incubated at 30 °C for between 5 h to 10 h. The colonies were treated with UV (30 wt, distance: 60 inch) for about 2 to 2.5 h. Surviving cells (about 5%) were then incubated at 30 °C overnight and then plated on agar plates having minimal medium containing nitrogen source, minerals, and

salts. 20 μ L toluene as a sole carbon source was added and the cultures were incubated at 30 °C for at least two days. The largest colonies were scored on the basis of size and collected. These colonies were considered fast-growing variants of toluene-metabolizing KR-1.

- 5 One such variant was isolated and termed KRC50. KRC50 was again subjected to mutagenesis by U.V. light and a further isolation of variants was conducted. The second set of variants produced KRC16 (ATCC 55886), which also metabolized toluene and has a growth rate higher than either KR-1 or KRC50.

10 Isolation of a *pobA* hybridization probe

The *pobA* gene from *P. mendocina* was obtained by PCR for use as a hybridization probe in the isolation of *pobA* from KRC16 and KR-1.

- The primers 5'-CARTTRCTNCAYAANGTNGGNAT-3' [SEQ ID NO:9] and 5'-CTAYTCNATYTCYTCRTANRG-3' [SEQ ID NO:10], based on the
15 published sequence of the *pobA* gene from *P. fluorescens* [SEQ ID NO:5] (See, van Berkel, W.J.H. et al., *supra*) were used in a PCR reaction consisting of 100 μ L reaction mixture which contained: 0.5 mM dNTPs, reaction buffer (final concentration of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, and 0.01% gelatin), 0.1 μ g of *P. mendocina* genomic DNA, 1 unit of Taq[®] DNA
20 polymerase.

- The DNA sample was denatured at 94 °C for 1 min, and annealed at 45 °C for 2 min. Polymerization was done at 72 °C for 3 min with an increased extension time of 5 sec per cycle. The polymerase chain reaction was accomplished by 30 cycles. The PCR DNA fragment was detected and analyzed
25 by regular 1% agarose gel electrophoresis with ethidium bromide. A 1.2 kb PCR DNA fragment containing the *pobA* gene was isolated and cloned into the vector of PCRscript (Pharmacia Biotech, Piscataway, NJ).

Preparation of genomic DNA from *P. mendocina* for isolation of *pobA-1*

- 50 mL of culture medium was used for the isolation of *P. mendocina*
30 genomic DNA. The cells of an overnight culture were spun down at 6000 rpm, 4 °C for 10 min. Supernatant was decanted and the pellets were resuspended with 5 mL TEG (25 mM Tris-HCl, 10 mM EDTA, 50 mM glucose, pH8). About 1.5 mL of RNase (100 μ g/mL) was added into the mixture. The sample was kept at room temperature for 5 min, and then extracted with phenol twice. The two
35 phases were separated by a centrifugation at 6000 rpm for 10 min. The aqueous phase was extracted with phenol:chloroform twice. Two volumes of 100% ethanol was added to the aqueous phase to precipitate DNA. After 20 min the solution was centrifuged at 10,000 rpm, and the pellet was collected, dried, and resuspended in about 2 to 5 mL TE buffer (10 mM Tris-HCl, 1 mM EDTA,

pH 7.6). The DNA samples were then dialyzed against TE buffer at 4 °C overnight.

Identification of *pobA-1* gene from *P. mendocina* genomic DNA

10 µg of *P. mendocina* genomic DNA was used for Southern blot analysis using the *pobA* hybridization probe described above. DNA samples were first digested with EcoRI, HindIII and SphI and the digests were analyzed by 1% agarose gel electrophoresis. The DNA samples were then transferred to nitrocellulose membrane by denaturation and neutralization as described previously. The membranes were exposed to U.V. light for about 2 min to produce nucleic acid crosslinking. Prior to crosslinking the membrane had been prehybridized for about 1 to 2 h at 65 °C in a solution containing 5 X SSC, 0.1% (w/v) SDS, 0.5% (w/v) blocking reagent (DuPont, Wilmington, DE) and 5% (w/v) Dextran Sulfate.

For ease of identification the 1.2 kb DNA *pobA* probe was labeled with a fluorescein nucleotide in a 30 µL reaction mixture containing: a random primer, reaction buffer, fluorescein nucleotide mix and Klenow enzyme at 37 °C for 1 h. The labeled probe was then hybridized to the membrane-bound genomic DNA in the same buffer for 16 h at 65 °C.

After hybridization, the membrane was washed for 15 min in 2 X SSC, 0.1% SDS, followed by a second 15 min wash in 0.2 X SSC, 0.1% SDS at 65 °C. The membrane was then blocked for 1 h in buffer containing 0.5% blocking reagent and then incubated with antiluorescein HRP conjugate (1:1000) at room temperature for 1 h.

After the incubation the membranes were washed four times for 5 min with 0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, and incubated in a chemiluminescence reagent (Renaissance, nucleic acid chemiluminescent reagent, DuPont, Wilmington, DE) for 1 min at room temperature, and then exposed to the REFLECTION™ film (DuPont, Wilmington, DE). In this manner portions of the genomic DNA hybridizing to the *pobA* probe were isolated.

Cloning the *pobA-1* gene

A partial genomic DNA library of *P. mendocina* KR1 was prepared. Genomic DNA was restricted with SphI which produced fragments of about 3.0 kb. The fragments were recovered using the DNA preparation kit, GeneClean Bio101 (Vista, CA). The SphI fragments were ligated into a SphI digested pUC18 vector (Pharmacia Biotech, Piscataway, NJ) and the recombinant plasmids were used to transform Dh5-a *E. coli*. Analysis of the transformants by miniprep and mini agarose gel electrophoresis (Sambrook, *supra*) indicated that about 80% of plasmid DNA contained an insert of about 3.0 Kb in length. Plasmids containing the 3.0 kb insert were termed pUC3.0k.

Confirmation and Isolation of *pobA-1* with toluidine

The correct isolation of *pobA-1* was confirmed using a colorimetric assay essentially as described by Parke ("Application of *p*-Toluidine in Chromogenic Detection of Catechol and Protocatechuate, Diphenolic Intermediates in Catabolism of Aromatic Compounds", *Applied and Environmental Microbiology*, Aug. 1992, 2694-2697).

Transformants containing the 3 kb insert were selected from the agar plates with ampicillin and were transferred onto the agar plates containing minimal medium, supplemented with 50 µg *p*-toluidine, 1 mM PHBA, 2.5 mM proline and 1 µg/mL thiamine and iron. The plates were incubated at 37 °C overnight.

Activity of *pobA-1* and the production of protocatechuate was confirmed by the appearance of a color on the plates.

Sequencing of the *pobA-1* gene

The plasmid of pUC3.0k *pobA* was used for DNA sequencing. The DNA sequencing with synthetic primers was done at The Biopolymer Laboratory, Department of Microbiology and Immunology at the University of Maryland, School of Medicine according to standard methods. Accordingly the sequence of *pobA-1* was determined and is given as SEQ ID NO:1.

EXAMPLE 2

CLONING OF *pobA-1* INTO A SUICIDE VECTOR AND TRANSFORMATION OF *P. MENDOCINA*

Cloning of the 3.0 kb *pobA* gene fragment into suicide vector pARO180

The 3.0 kb *pobA* gene fragment was isolated from pUC3.0k by digesting the plasmid with SphI and further isolation by gel electrophoresis. The isolated 3.0 kb restriction fragment of *pobA* gene was then ligated with the SphI digested mobilizable suicide vector pARO180 (Parke, *Gene* (1990), 93(1), 135-7) to produce pARO3.0pobA. Plasmid pARO180pobAΩ contains an omega fragment inserted into the HindIII site of the first *pobA* gene on a 3.0 kb SphI DNA fragment.

Prior to ligation the HindIII site adjacent to the SphI site on pARO180 was destroyed using a Klenow fragment (Figure 1(a)) pARO3.0pobA was then used to transform Dh5α *E. coli*.

Disruption of the *pobA-1* gene with Omega element

A 2.0 kb HindIII restriction fragment of omega element (Prentki et al., *Gene* 29, 303, (1984), was inserted into the HindIII site of pARO3.0pobA which is located within the coding region of the *pobA* gene. The resulting plasmid was designed as pARpobA omega.

Introduction of the disrupted *pobA* gene into *P. mendocina* strain KRC16

The mobilizing *E. coli* strain S17-1 (Parke et al., *supra*) was used to introduce the disrupted *pobA-1* gene containing the omega element into *P. mendocina* KR16 (Figure 2).

- 5 pARpobA omega containing the 3.0 kb omega-disrupted *pobA-1* was used to transform KRC16 using a standard transformation procedure as described below. A single colony of S17-1 strain having the plasmid of pobAomega was inoculated in 20 mL LB medium, and grown at 37 °C until log phase. Additionally untransformed *P. mendocina* strain KR16 was also inoculated in
- 10 20 mL LB medium and incubated at 30 °C until log phase. The cells of both strains were washed with LB medium two times and resuspended in LB medium. A mixture of donor and recipient cells [S17-1 cells and *P. mendocina* KR1] at a ratio of 1:4 was plated on the agar plates of LB medium. The plates were incubated at 30 °C for 8 h. The cells were collected and then plated on the agar
- 15 plates containing phosphate buffer, 1 mM succinate, 10 mM streptomycin. The colonies of *P. mendocina* strain which can grow on the selection plates were scored and collected. Positive transformants were designated KRC16::pobAΩ (Figure 2).

Identification of the disruption of *pobA-1*

- 20 Genomic DNA was isolated from wildtype and mutant KRC16::pobAΩ *P. mendocina* and restricted with SphI and then subjected to 1% agarose gel electrophoresis. After gel electrophoresis, the DNA samples were transferred to nylon membrane, and denatured and neutralized as described previously. The membranes were exposed to UV for about 2 min and the membrane was
- 25 prehybridized for about 1 to 2 h at 65 °C in a solution containing 5 X SSC, 0.1% (w/v) SDS, 0.5% (w/v) blocking reagent (DuPont, Wilmington, DE) and 5% (w/v) Dextran Sulfate.

- The 1.2 kb DNA fragment of *pobA-1* (Example 1) was labeled with fluorescein nucleotide in a 30 µL reaction mixture containing random primer,
- 30 reaction buffer, fluorescein nucleotide mix and Klenow enzyme at 37 °C for 1 h. Hybridization to labeled probe (16 h at 65 °C) was done by using the above-described solution.

- After hybridization, the membrane was given one 15-min wash in 2 X SSC, 0.1% SDS, and one 15-min wash in 0.2 X SSC, 0.1% SDS (65 °C). The
- 35 membrane was blocked for 1 h in buffer of 0.5% blocking reagent and then incubated with anti fluorescein HRP conjugate (1:1000) at room temperature for 1 h. After the membrane was washed for 5-min. with buffer of 0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, it was incubated in the chemiluminescence reagent for 1 min at room temperature, and then exposed to the REFLECTION™ film.

Analysis of the exposed film confirmed the presence of the disrupted *pobA-1*, seen as a shift in the mobility from the 3.0 kb fragment of the wildtype.

EXAMPLE 3

ANALYSIS OF PHBA PRODUCTION IN *pobA-1(-)* *P. MENDOCINA*

5 Example 3 demonstrates the conversion of toluene to PHBA by *P. mendocina* strains KRC16::*pobA* Ω , and KRC16.

Organisms were initially grown on LB agar plates containing (gr): tryptone, 10.0; yeast extract, 5.0; sodium chloride, 10.0; agar Noble, 15.0; double distilled H₂O, 1.0 liter (pH ~6.6). Plates were incubated for ~60 h at 30 °C and
10 were then stored at 4 °C. One liter flasks (x20) each 125 mL center-well flask contained 25 mL of the PHBA medium containing (gr): sodium phosphate, monobasic, 1.85; potassium phosphate, dibasic, 5.84; ammonium sulfate, 1.50; glucose, 1.0; yeast extract, 0.25; tryptone, 0.25; double distilled water, 1.0 liter; (pH 7.0-7.2) and 250 μ L of trace minerals. The trace minerals solution contained
15 the following which were added to ~50 mL of double distilled H₂O containing 1.0 mL of concentrated HCl, (in mg): MgCl₂, 6H₂O, 2.50; CaCl₂, 2H₂O, 1.00; MnCl₂, 4H₂O, 400; Na₂MoO₄, 2H₂O, 750; FeSO₄, 7H₂O, 100; CuSO₄, 5H₂O, 100; ZnSO₄, 7H₂O, 100. The volume was adjusted to 100 mL. Trace mineral solution (1.0 mL) was added to 100 mL of the minimal medium after autoclaving.
20 Toluene (500 μ L) was added to the center-well. Cells were grown for 24 h. Culture suspensions (5 mL) were then used to inoculate 200 mL of the fresh medium in one liter flasks. These were incubated with shaking for an additional 24 h. Culture supernatant (50 mL) of each strain was acidified to pH 2-3 and extracted (x3) with ethylacetate (50 mL), dried over magnesium sulfate and
25 evaporated to dryness under a stream of nitrogen. Samples were resuspended in ethylacetate (500 μ L) and BSTFA (500 μ L) was added. The vials were capped and heated to ~80 °C for 30 min to allow for complete derivatization. Samples were then transferred to GC vials and the volume was adjusted to 1.0 mL and were analyzed by GC as outlined above.

30 GC analysis showed the presence of PHBA in cultures of KRC16::*pobA* Ω cells grown on toluene. This sample was then submitted for GC/MS analysis for further confirmation of the presence of PHBA and PCA.

EXAMPLE 4

DETERMINATION OF THE PRESENCE OF AN ALTERNATE *pobA* GENE

35 Conversion of PHBA to Protocatechuate by *P. mendocina* Strains KR16::*pobA* Ω and KR1 Following Growth on *p*-Cresol

Since strain KR1::*pobA* Ω contains the interrupted *pobA* gene, this organism is expected to be incapable of converting PHBA to protocatechuate (PCA).

Organisms were grown on Laura Broth agar (LB) plates. Flasks (8 x 125 mL) each containing 25 mL of PHBA minimal medium were set up and *p*-Cresol (1 mM filter-sterilized). Flasks were inoculated with colonies from 1.5 plates per flask and were incubated at 30 °C on the shaker (200 RPM) for ~24 h. Cells obtained, (KR16*pobA*Ω, 6.26 g, and KR1, 6.37 g) were then stored at -80 °C until needed. The PEG buffer contained: 50 mM sodium phosphate buffer plus 10% glycerol, 1.0 mM DTT and 0.2 mM MgCl₂. Cells were then disrupted by passing twice through the French Pressure cell (3/8", 20,000 PSI). The cell debris was removed by centrifugation for 25 min at 38,000 x g at 4 °C. PMSF (phenylmethylsulfonyl flouride) was added for a final concentration of 200 micro Molar after the cells were disrupted. The second pass through the French Pressure Cell was followed with a 3.0 mL rinse with PEG buffer. The protein concentration in the crude extract was determined using the Bicinchoninic acid (BCA) method as described by the manufacturer (Pierce, Rockford, IL).

15 The Effect of Buffers on the Rate of PHBA Hydroxylation

To study the effect of various buffers on the rate of PHBA hydroxylation, two separate buffers, Tris/HCl pH 8.0 and 50 mM sodium phosphate pH 6.8 buffers were used. The incubation mixtures for conversion of PHBA to PCA contained: NADPH, 6.0 μmoles; PHBA, 11.6 μmoles; FAD, 1.0 μmole and 2.0 mg protein of each extract (KR16::*pobA*Ω and KR1) and buffer to a total volume of 5 mL. After protein addition, the reaction mixtures were incubated with shaking at 30 °C for 5 min, stopped by the addition of 2 drops concentrated HCl, and extracted (x3) with diethyl ether (5 mL). The organic pool was dried over Na₂SO₄ and evaporated to dryness under a stream of nitrogen. The data is summarized below in Table 1.

TABLE 1

Organism/Buffer	PCT formed μg/mL	PCA formed nmole/mL
KR16:: <i>pobA</i> Ω	22	143
Na-phosphate		
KR1	12.5	81
Na-phosphate		
KR1:: <i>pobA</i> Ω	18.7	121
Tris-HCl		
KR1 Tris-HCl	18.8	122

As can be seen by the data in Table 1, the PHBA hydroxylase is still active in KR16::*pobA*Ω because PCA is being formed from PHBA by this organism at levels comparable to the wild type (KR1) organism. No significant difference was observed in the amount of PCA formed with either phosphate or Tris buffer.

Conversion of PHBA to PCA by *P. mendocina* strains, KRC16::pobA Ω , KRC16, and KR1 when grown on *p*-Cresol

Since strain KRC16::pobA Ω , KRC16, and KR1 contain the interrupted *pobA* gene, these organisms are expected to be incapable of converting PHBA to PCA.

Using a single loop of colonies from the agar plates, flasks containing *p*-cresol (1 mM), were inoculated and allowed to grow with shaking for 24 h at 30 °C. These cultures were used to inoculate 200 mL of medium containing *p*-cresol (1.0 mM). Organisms were harvested after 24 h, washed, and the crude extract was obtained as described above.

Conversion of PHBA to PCA

Incubation mixtures contained: NADPH, 6.0 μ moles; PHBA, 11.6 μ moles; FAD, 1.2 μ moles; crude extract, 2.0 mg; Tris/HCl, pH 8.0 to final volume of 5.0 mL. Reaction mixtures were incubated for 5 min at 30 °C on shaker and were then stopped with 2 drops of concentrated HCl. The contents of the vials were extracted and prepared for GC analysis as explained above. Tests were performed in duplicates. Table 2 summarizes the data obtained.

TABLE 2

Organism	PCA formed μ g/mL	PCA formed μ moles/mL
KRC16::pobA Ω	19.5	127
KRC16	24.3	159
KR1	21.6	141

As can be seen by Table 2, PHBA hydroxylase enzyme is active in all three organisms. KRC16::pobA Ω , in which the *pobA* gene was interrupted, is still capable of converting PHBA to PCA. This data implies the presence and functioning of more than one PHBA hydroxylase encoding gene (*pobA*) in *P. mendocina* KR1.

Effect of EDTA on PCA Accumulation in Cultures

The ability of *P. mendocina* strains KRC16::pobA Ω , KRC16, and KR1 to convert PHBA to PCA when grown on *p*-cresol was verified and the effect of ethylenediaminetetraacetic acid (EDTA) on the accumulation of PCA was studied. EDTA was expected to prevent further degradation of PCA and thus allow for its accumulation. Organisms were grown on *p*-Cresol (1.0 mM) and the crude extract was obtained as described before.

The reaction mixtures, which were set up in duplicates, contained: NADPH, 6.0 μ moles; PHBA, 11.6 μ moles; FAD, 1.2 μ moles; EDTA, 3.0 μ moles;

protein, 2.0 mg; sodium phosphate buffer (50 mM) pH 6.8 to final volume of 5.0 mL.

Reaction mixtures were incubated for 5 min at 30 °C on the shaker and were terminated with 2 drops of concentrated HCl. Contents of the vials were
5 extracted and prepared for GC analysis as before. Table 3 summarizes data for analysis.

TABLE 3

Organism	PCA formed µg/mL	PCA formed µmoles/mL
KRC16:: <i>pobA</i> Ω	9.9	64
KRC16:: <i>pobA</i> Ω (-EDTA)	9.3	61
KRC16	19.9	78
KR1	15.1	98

As can be seen in Table 3, PHBA hydroxylation is active in all organisms,
10 and addition of EDTA resulted in about 5% increase in the amount of PCA formed.

EXAMPLE 5

CLONING AND SEQUENCING OF *pobA-2*

Cloning of *pobA-2* gene

15 A partial genomic DNA library of *P. mendocina* KRC16, was constructed as follows. Genomic DNA was restricted with SphI and 4.5 kb restriction fragments were isolated and recovered from an agarose gel using a DNA preparation kit, (GeneClean Bio101, Vista, CA). Isolated DNA was then ligated into pUC18 at the appropriate SphI restriction sites and used to transform *E. coli*
20 Dh5α.

Miniprep and mini agarose gel electrophoresis indicated that about 80% of the transformants contained an insert of about 4.5 Kb in length.

Identification and isolation of *pobA-2* by colony hybridization

25 The positive transformants were first grown on the LB agar plates overnight. The plates were chilled to 4 °C for one h. The colonies were then transferred onto the membrane discs. The membrane discs were treated with 0.5 N NaOH twice 2-min, and then washed twice for 2-min with 1 M Tris-HCl, pH 7.5. The DNA was fixed on the membrane by UV crosslinking. Membranes were prehybridized for about 1 to 2 h at 65 °C in a solution containing 5 X SSC,
30 0.1% (w/v) SDS, 0.5% (w/v) blocking reagent (DuPont, Wilmington, DE) and 5% (w/v) Dextran Sulfate.

The 1.2 kb DNA fragment of *pobA* gene, constituting the *pobA* hybridization probe, was labeled and used to detect the presence of *pobA-2* in

essentially the same manner as it was used to confirm the presence of *pobA-1* as described in Example 1.

The plasmid DNA was isolated from the positive colony detected with colony hybridization. The size of the insert was determined by the SphI digestion and miniagarose gel electrophoresis.

Sequencing of the *pobA-2* gene

The plasmid of pUC4.5k *pobA* was used for DNA sequencing. The DNA sequencing with synthetic primers was done at The Biopolymer Laboratory, Department of Microbiology and Immunology at the University of Maryland, School of Medicine according to standard methods. Accordingly the sequence of *pobA-2* was determined and is given as SEQ ID NO:2.

EXAMPLE 6

CONSTRUCTION OF THE DISRUPTED *pobA-2* AND CLONING INTO A SUICIDE VECTOR

Disruption of *pobA-2* gene by deletion and insertion

The Kam resistance gene cassette was removed from the plasmid pUC4K (Pharmacia Biotech, Piscataway, NJ) by digesting with EcoRI (Figure 1(b)). Sticky ends were then repaired with Klenow. pK4.5pobA, containing the 4.5 kb fragment encoding pobA was then digested with Eco47III, which resulted in a Eco47III fragment being deleted from the coding region of *pobA-2* on the 4.5 kb SphI fragment (Figure 1(b)).

The Kam resistance gene cassette was then inserted into the Eco47III digested pK4.5pobA plasmid to form the plasmid of pK4.5pobA-Kam. The orientation of the Kam gene cassette in the pobA gene was determined by the restriction mapping of the resulted plasmid.

Cloning of the disrupted *pobA-2* gene into suicide vector

The disrupted *pobA-2* gene with Kam gene cassette was digested with SphI and the resulting DNA fragment was ligated with the SphI digested pARO180 plasmid to produce pAR04.5pob-K, which was then used to transform S17-1 *E. coli* (Figure 1(b)). Positive colonies were selected on the agar plates on the basis of Kam resistance.

Introduction of the disrupted *pobA-2* gene into the mutant of *P. mendocina* KRC16::*pobA*Ω

A single colony of S17-1 strain having the plasmid of pARP4.5pobA-Kam (Figure 1(b)) was inoculated in 20 mL LB medium, and grown at 37 °C to log phase. Another colony of KRC16::*pobA*Ω was inoculated in 20 mL LB medium and incubated at 30 °C and grown to log phase.

The cells of both cultures were washed twice with LB medium and resuspended in LB medium. S17-1 cells and *P. mendocina* KRC16::*pobA*Ω were

mixed at a ratio of 1:4 and were plated on agar plates of LB medium. The plates were incubated at 30 °C for 8 h. The cells were collected and then plated on the agar plates containing phosphate buffer, 1 mM succinate, 10 mM streptomycin and 25 µg/mL Kanamycin, and Kan-resistant colonies were selected.

5 Selection *P. mendocina* having a disruption in both *pobA-1* and *pobA-2*

A set of agar plates containing various substrates were used to select the mutants. The substrates were toluene, 20 µL for 30 mL medium, 1 mM *p*-cresol, 1 mM PHBA or 1 mM PCA respectively. The colonies were plated on these four plates containing different substrates as sole carbon source. Only the colonies of the mutants which cannot grow in the presence of PHBA and either toluene or *p*-cresol were scored while all of the colonies were grown with PCA. A similar experiment for further confirmation of the mutants was also conducted with liquid minimal medium containing the various substrates such as toluene (200 µL for 20 mL medium), 1 mM *p*-cresol, 1 mM PHBA or 1 mM PCA. The cell density was monitored with a spectrophotometer. The mutants which did not utilize toluene and PHBA in the liquid minimal medium were identified as the *P. mendocina* strain having a disruption of both *pobA-1* and *pobA-2* genes.

15 Identification of the disruption of *pobA-1* and *pobA-2* genes the *pobA(-)*

P. mendocina strain

20 Genomic DNA was isolated from the mutant strain of KRC1651 (ATCC 55885). DNA from both wild type and mutant cells were digested with SphI and then subjected to 1% agarose gel electrophoresis. After gel electrophoresis, the DNA samples were transferred to nylon membrane, and denatured and neutralized as described previously. The membranes were exposed to the UV for about 2 min for UV crosslinking of nucleic acids. Membranes were prehybridized for about 1 to 2 h at 65 °C in a solution containing 5 X SSC, 0.1% (w/v) SDS, 0.5% (w/v) blocking reagent (DuPont, Wilmington, DE) and 5% (w/v) Dextran Sulfate. The 1.2 kb DNA fragment of *pobA* gene was labeled with fluorescein nucleotide in a 30 µl reaction mixture containing random primer, reaction buffer, fluorescein nucleotide mix and Klenow enzyme at 37 °C for 1 h. Hybridization to labeled probe (16 h at 65 °C) was done by using the above-described solution. After hybridization, membrane was given one 15-min wash in 2 X SSC, 0.1% SDS, and one 15-min wash in 0.2 X SSC, 0.1% SDS (65 °C). The membrane was blocked for 1 h in buffer of 0.5% blocking reagent and then incubated with antifluorescein HRP conjugate (1:1000) at room temperature for 1 h. After the membrane was washed four 5-min with buffer of 0.1 M Tris-HCL pH 7.5, 0.15 M NaCl, it was incubated in the chemiluminescence reagent for 1 min at room temperature, and then exposed to the REFLECTION™ film.

EXAMPLE 7PRODUCTION OF PHBA WITH *pobA* (-) *P. MENDOCINA*Conversion of Toluene to PHBA by the *pobA*(-) mutants *P. mendocina* Strains KRC1615, KRC1616 and KRC1651

5 Example 7 was designed to determine the ability of *P. mendocina* KRC1615, KRC1616 and KRC1651 *pobA*(-) mutants to convert toluene to pHBA when grown on succinate. The PHBA minimal medium with the following changes was used throughout the experiment: toluene, glucose, and tryptone were omitted and sodium succinate (at 1% final conc.) and yeast extract (at 0.01% final
10 conc.) were added (pH 7.1). Cells were harvested and washed after 21 h of growth. Center-well flasks were set up with the following additions: medium, 22.0 mL; trace mineral, 250 µL; toluene, 750 µL (center-well only); organisms, 300 mg (wet weight). Flasks were incubated with shaking at 30 °C. Samples were prepared for GC analysis as before. GC/MS analysis was performed and
15 results are attached as Figure 5.

As can be seen in Figure 5, no PCA was detected in any samples. PHBA was detected in some incubations after 50 h (strains KRC1615, KRC1616 and KRC1651) and after 95 h (KRC1616) as indicated below in Table 4:

20

TABLE 4

Strain	Time (h)	µg PHBA formed
KRC1615	50	39.5
KRC1651	50	770
KRC1651*	50	4500
KRC1616	95	39.8
KRC1616*	95	58.8

* Indicates duplicates

As demonstrated by the data in Table 4 strain KRC1651 was most active in PHBA production.

25 These data indicate that strain KRC1651 is capable of converting toluene to PHBA and accumulating it. This implies that the pathway to form PCA from PHBA has been blocked in this organism. Further experiments on PHBA accumulation were therefore planned using *P. mendocina* KRC1651.

30 Effect of Glucose, Succinate and MgCl₂ on Growth and PHBA Production by the *pobA*(-) mutant KRC1651

The center-well flasks containing 200 mL of Luria Broth (LB) medium: Tryptone (1%); yeast extract (0.5%); sodium chloride (1%); double distilled water up to 1.0 l. One mL of a filter sterilized solution of kanamycin (4.0 mg/1 mL)

was added to each flask after cooling. Flasks were inoculated with KRC1651 and incubated with shaking at 30 °C. After 24 h, 1.0 mL of toluene was added to the center-well and incubation was continued for one more h. Organisms were harvested and the pellets were washed 2x with phosphate buffer (pH 7.0). Final centrifugation at 17,000 x g for 30 min resulted in 4.34 g wet weight of cells. Center-well flasks containing the PHBA minimal medium in which yeast extract was replaced with 1.0 mM of glucose or 1.0 mM of sodium succinate were inoculated with 620 mg (wet weight) of cells. Varying concentrations of MgCl₂ (1.2 mM - 5.0 mM) were used in these experiments. Toluene (2.0 mL, center-well) was added and the flasks were incubated with shaking at 30 °C. Flasks with standard PHBA trace mineral solutions were sampled at 2, 5, 24, 48 and 68 h. Flasks with increased MgCl₂ concentration were sampled at 2, 48 and 68 h. These were extracted, derivatized and analyzed as described above. The results of these analysis are summarized below in Tables 5 and 6.

TABLE 5

Glucose Medium

<u>Sample</u>	<u>PHBA formed/mL</u>	<u>Sample</u>	<u>PHBA formed/mL</u>
Standard-2	13.8 µg	Increased MgCl ₂ -2	11.6 µg
Increased MgCl ₂	24.16 µg	Increased MgCl ₂ -48	0.08 mg
Standard-24	0.11 mg	Increased MgCl ₂ -68	---
Standard-48	0.44 mg		
Standard-68	0.04 mg		

TABLE 6

Succinate Medium

<u>Sample</u>	<u>PHBA formed</u>	<u>Sample</u>	<u>PHBA formed</u>
Standard-2	32.5 µg	Increased MgCl ₂ -2	9.8 µg
Increased MgCl ₂	38.8 µg	Increased MgCl ₂ -48	26.4 µg
Standard-24	0.07 mg	Increased MgCl ₂ -68	22.48 µg
Standard-48	35.9 µg		
Standard-68	33.64 µg		

As can be seen by the data, PHBA concentrations decreased after 24 h, however no PCA was detected in samples. Further, the higher concentrations of MgCl₂ did not increase the ability of organisms to transform toluene to PHBA.

Conversion of PHBA to PCA by Crude Extracts of KRC1651 and KRC16

Purpose: to determine the ability of *P. mendocina* strains KRC1651 (ATCC 55885) and KRC16 to convert PHBA to PCA. The cells obtained above were disrupted as described before and the crude extract obtained was used for

experiments described above. The reaction mixture contained: NADPH⁺, 6.0 μ moles; PHBA, 11.6 μ moles; FAD, 1.2 μ moles; EDTA, 3.0 μ moles; protein, 2.0 mg; sodium phosphate buffer (50 mM) to final volume of 2.0 mL. To insure that the PCA formed was not further degraded, ethylenediaminetetraacetic acid (EDTA) was used as an inhibitor of protocatechuate dioxygenase. The reaction mixture was incubated with shaking for 30 min at 30 °C after which it was terminated by acidifying to <pH 2 followed by addition of 10 mL ethylacetate. This mixture was then allowed to remain at room temperature for approximately 16 h. It was then further extracted (x2) using 5 mL of ethylacetate. The organic layer was dried over Na₂SO₄ and then evaporated to dryness under a stream of nitrogen. Samples were prepared for GC analysis as before and results are summarized below in Table 7:

TABLE 7

Sample	PHBA Detected	PCA Formed per Reaction
KRC1651	0.88 mg	---
KRC-16	0.86 mg	3.9 μ g

As can be seen by the data, no PCA was detected by GC analysis of the *pobA*(-) strain *P. mendocina* KRC1651 (ATCC 55885). Further, the strain containing the *pobA* gene (*P. mendocina* KRC16) showed formation of 3.9 μ g of PCA indicating that it is converting PHBA to PCA.

EXAMPLE 8Effect of glucose concentration on PHBA production

Example 8 demonstrates the effect of glucose on cell yield and the production of PHBA from KRC1651 (ATCC 55885).

Cell Yield - Fed Batch

Cell yield was tested in the mineral medium with 2 g/L glucose and 0.48 g/L yeast extract. The cell yield obtained was corrected for yeast extract contribution and was the highest on fructose and the lowest on succinate, >0.38 and >0.22 gr cell/gr substrate, respectively. Yeast extract, peptone, casamino acids, tryptone, N-Z-amine E, corn steep liquor, and soytone were tested as organic nitrogen additives to the similar medium. All additives stimulated growth and cell yield. The growth rate was influenced by temperature, medium additions and the density of the culture. The initial growth rates at low cell densities were maximal at 34-36 °C with doubling times of 35 and 33 min. respectively. At higher cell densities, the growth rate was much slower and was estimated in fermenters, at 10 gr cells/L to have a doubling time of about two hours. The final cell densities on lean medium were limited to <4.7 g/L, Fed-Batch studies with

addition of glucose, ammonia, yeast extract and N-Z-amine E achieved 40.0 g/L. Cell yield based on glucose ranged from 0.22 to 0.47 g/g in the lean medium to 0.41 to 0.60 in the rich medium.

Cell Yield - Continuous Culture

- 5 Using glucose as a carbon source, *P. mendocina* demonstrated an ability to grow in continuous culture. Cultures were prepared with 1-L SixFour fermenter with mineral medium, 10 g/L glucose, 0.75 g/L (NH₄)₂SO₄ and 0.28 g/L yeast extract and at dilution rates ranging from 0 to 0.3 h (2.5 h doubling time). The cell yield in these cultures was 0.3 g/g.

10 Production of PHBA in Continuous Culture

The specific productivity of PHBA was tested at 1.25 to 4 g/L cell concentrations and 4 h incubation. Maximal productivity occurred at 0.125-0.5 g/L and it progressively decreased at 1 and 2 g/L.

EXAMPLE 9

15 Effect of pH on PHBA production

The effect of pH was determined on KRC1651 (ATCC 55885) grown in both rich and lean medium according to the conditions recited in the GENERAL METHODS.

- 20 pH had a significant effect on PHBA production, where increasing the pH from 6.3 to 7.4 increased PHBA specific productivity by about two fold. At higher pH values the specific productivity of PHBA varied with the medium used. Lean medium produced no change in PHBA specific productivity in a pH range of 7.2 to 7.9, while in rich medium the specific productivity increased by about 4 fold. The pH of the cultures should be maintained within a range of about from
25 6.3 to 7.4, where a range from about 7.2 to 7.7 is most preferred.

EXAMPLE 10

Effect of toluene and PHBA concentration on the production of PHBA

- 30 Example 10 demonstrates the effect of toluene concentration on the production of PHBA from KRC1651 (ATCC 55885). Two separate protocols were used for the analysis, one in 125 ml Culture flasks and the other under conditions of batch fermentation in a continuous feed bioreactor.

Culture Flasks

- 35 Growth studies using KRC1651 (ATCC 55885) were performed in 125 mL sealed flasks with 5 mL minimal medium plus 0.28 g/L yeast extract and 10 g/L glucose. The flasks were incubated at 30 °C in incubator shaker. Growth was observed after 24 and 48 h visually or by OD measurement at 600 nm.

Mutants with higher tolerance to toluene and PHBA were selected by repeated transfers in media with increasing concentrations of toluene and PHBA.

Repeated transfers produced strains that could grow on agar plates at 40 g/L PHBA and about 100 ppm toluene.

Both toluene and PHBA have a bacteriostatic and bacteriocitic effects. The growth of KRC1651 was significantly inhibited at about 100 ppm toluene or 10 g/L PHBA. Together these agents had an additive toxic effect.

Bioreactor

KRC1651 (ATCC 55885) was used in bioreactor studies and grown in rich and lean medium. Production of PHBA from toluene can be enhanced by a continuous feed of toluene to a bioreactor to keep the steady state concentration of toluene in the reactor below toluene explosivity limits. The lower explosivity limits (LEL) for toluene is 120 ppm in the liquid phase, 1.2% v/v in the gas phase. Glucose was fed as a co-substrate for cell maintenance simultaneously with toluene feed. The standard system used consisted of 1 mM glucose, 60 ppm toluene, 0.25-0.5 g cells/L, and the incubation was for 3-4 h. The pH was controlled at 7.2 with addition of buffer. The effect of toluene concentration on PHBA production was examined at 30, 60, and 100 ppm.

The time course of PHBA production was followed from 0 to 24 h. PHBA production (based on accumulation) demonstrated a lag of 1 to 2 h where maximal productivity was at about 2-3 h. At later times a decline from 4 to 6-10 h and zero production from 6-10 h to 24 h was seen. No decline in PHBA concentration was observed. Results were comparable between rich and lean medium; however, in the rich medium the PHBA productivity was somewhat higher reaching a maximum of 60 ppm.

EXAMPLE 11

Production of PHBA with *pobA* (-) *P. mendocina* - Effect of Mg+ on Toluene Tolerance

Toluene and PHBA are known to incorporate and interfere with the integrity of bacterial membranes and function. Growth studies in the presence of inhibitory concentrations of toluene and PHBA were used to assess the protective effect of Mg ions.

Experimental: Growth studies using KRC1651 (ATCC 55885) were performed in 125 mL sealed flasks with 5 mL minimal medium plus 0.28 g/L yeast extract and 10 g/L glucose. The flasks were incubated at 30 °C in incubator shaker. Growth was observed after 24 and 48 h visually or by OD measurement at 600 nm.

Results: Magnesium ions increased KRC1651 tolerance to toluene and PHBA. Increasing Mg concentration from 2 to 12 mM enhances growth rate at 100 and 150 ppm toluene and allows growth at 100 ppm toluene and 5 g/L PHBA.

EXAMPLE 12Maximal production rates of PHBA

KRC1651 (ATCC 55885) was cultured in rich medium where the growth conditions of pH, glucose, Mg ion concentration and toluene were optimized for maximal PHBA production as described in Examples 8-11. With all conditions optimized, PHBA production varied with the method of production. The most apparent difference was between cells that grew in fermenters and flasks. Cells that grew in fermenters had an average specific productivity of 0.35 g/g-h and cells that grew in flasks had an average specific productivity of 0.11 g/g-h. The maximal specific productivity in fermenters occurs at 1-3 h and is estimated to be 1 g/g-h.

EXAMPLE 13Fed-Batch Fermentation of Toluene to PHBA

A two-step fermentation was carried out to demonstrate the production of PHBA from toluene using the *P. mendocina* KRC16KDpobA51 strain. This strain had disrupted *pobA-1* and *pobA-2* genes. The fermentation was carried out in two steps:

1. biomass built-up step (batch process), and
2. biotransformation step (fed-batch process)

During the first step, the *P. mendocina* KRC16KDpobA51 strain grew to high cell densities in a 5 L fermenter with approximately a 3 L working volume. The medium contained 1.2 g KH_2PO_4 , 6 g $(\text{NH}_4)_2\text{SO}_4$, 0.3 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20 mL/L trace elements, 20 g/L glucose, 20 g/L corn steep liquor, 1 mL/L polypropylene glycol and 1 g/L yeast extract. The trace elements contained 10 g/L citric acid, 1.5 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2.8 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.39 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.38 g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.2 g/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, and 0.3 g/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$. The phosphate, ammonia, Mg salts and trace elements were sterilized together and then titrated to pH 7.2 with KOH or H_2SO_4 before addition of the remaining components.

The second step commenced as a toluene fed-batch fermentation using glucose as co-feed at a rate of 3 g/h. The rate was decreased if glucose concentration rose above 1 g/L. Toluene was supplied at a rate that kept its concentration in the medium at 10-100 ppm. The initial cell density was 16.2 OD units. Over 18.5 h, a total of 67.3 g (0.73 moles) of toluene was fed during step 2 of the biotransformation. All of the toluene was not utilized and some was lost with the air exit-stream of the fermenter. After 18.5 h, a total of 38.1 g PHBA (0.278 moles) was produced. Table 8 summarizes the production of PHBA over time.

TABLE 8

Time (h)	PHBA (g/L)
0.0	0.0
1.0	0.0
1.4	0.0
2.4	0.7
3.4	1.7
4.5	3.4
5.4	6.1
6.4	7.6
7.3	6.9
8.3	10.7
9.3	9.1
10.5	8.9
11.5	9.1
12.7	10.7
14.5	9.7
15.5	12.1
17.0	11.4
17.4	12.6
18.5	12.6
18.5	12.7

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT:
 - (A) ADDRESSEE: E. I. DU PONT DE NEMOURS AND COMPANY
 - (B) STREET: 1007 MARKET STREET
 - (C) CITY: WILMINGTON
 - (D) STATE: DELAWARE
 - (E) COUNTRY: U.S.A.
 - (F) ZIP: 19898
 - (G) TELEPHONE: 302-982-8112
 - (H) TELEFAX: 302-773-0164
 - (I) TELEX: 6717325
- (ii) TITLE OF INVENTION: METHOD FOR THE PRODUCTION OF
PARA-HYDROXYBENZOATE IN
PSEUDOMONAS MENDOCINA
- (iii) NUMBER OF SEQUENCES: 10
- (iv) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: DISKETTE, 3.50 INCH
 - (B) COMPUTER: IBM PC COMPATIBLE
 - (C) OPERATING SYSTEM: MICROSOFT WINDOWS 95
 - (D) SOFTWARE: MICROSOFT WORD VERSION 7.0A
- (v) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vi) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 60/049,556
 - (B) FILING DATE: JUNE 13, 1997
- (vii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: FLOYD, LINDA A.
 - (B) REGISTRATION NUMBER: 33,692
 - (C) REFERENCE/DOCKET NUMBER: CR-9967-A

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1140 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CGCACCGACC TGCTGGAGAA GTATTCGGAG CTGGCTCTAC GCCGCGTGTG GAAAGGTGAA 1020
CGCTTCAGCT GGTTCATGAC CAATCTGCTG CACGACTTCG ACGGTCACCA GGACGCCTGG 1080
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(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 21 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
 (A) DESCRIPTION: /desc = "PRIMER"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GRNATRCTYC TYTANCTYAT C

21

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1188 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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GGCAAGCGGG TGCCGGTCCA CCTTACCGAG CTGACCGGTG GCAAGAGCGT GATGGTCTAT  300
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TATCCCTTCG GCTGGCTGGG CCTGCTCTCC GACACCCCGC CGGTGCATGA GGAATCATC  600
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GACCAGAAGA TGCAGCAGGC CGACCGCGAG TACTTCCTCG GTTCCCACGC GGGCCTCGTG 1140
AACATCGCCG AGAACTACGT GGGCCTGCCC TACGAAGACG TGAAATAA           1188
  
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(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 268 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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 35 40 45
 Glu Gln Gly Thr Val Asp Met Leu Arg Glu Ala Gly Val Ala Gln Arg
 50 55 60
 Met Asp Ala Glu Gly Leu Val His Glu Gly Val Glu Leu Val Phe Asp
 65 70 75 80
 Gly Lys Arg Val Pro Ile His Leu Lys Ala Leu Thr Xaa Xaa Xaa Xaa
 85 90 95
 Xaa Xaa Gly Gly Lys Thr Val Met Val Tyr Gly Gln Thr Glu Val Thr
 100 105 110
 Arg Asp Leu Met Asp Ala Arg Val Ala Val Gly Ala Pro Ile Val Tyr
 115 120 125
 Ser Ala Glu Asn Val Gln Leu His Glu Leu Lys Gly Gly Lys Pro Tyr
 130 135 140
 Val Thr Phe Glu Lys Asp Gly Gln Ser His Arg Ile Asp Cys Asp Tyr
 145 150 155 160
 Ile Ala Gly Cys Asp Gly Phe His Gly Ile Ala Arg Lys Ser Ile Pro
 165 170 175
 Ala Gly Val Leu Thr Glu Tyr Glu Arg Val Tyr Pro Phe Gly Trp Leu
 180 185 190
 Gly Leu Leu Ser Asp Thr Pro Pro Val His Glu Glu Leu Ile Tyr Ala
 195 200 205
 His His Glu Arg Gly Phe Val Leu Cys Ser Gln Arg Ser Leu Thr Arg
 210 215 220
 Ser Arg Tyr Tyr Leu Gln Val Pro Leu Thr Asp Lys Val Glu Asp Trp
 225 230 235 240
 Ser Asp Glu Arg Phe Trp Asn Glu Leu Lys Ala Arg Leu Pro Gln Asp
 245 250 255
 Val Ala Asp Arg Leu Val Thr Gly Pro Ser Leu Glu
 260 265

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 395 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: not relevant
 - (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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 20 25 30

Arg Gln Thr Pro Glu Tyr Val Leu Gly Arg Ile Arg Ala Gly Val Leu
 35 40 45
 Glu Gln Gly Thr Val Asp Met Leu Arg Glu Ala Gly Val Ser Glu Arg
 50 55 60
 Met Asp Ala Glu Gly Leu Val His His Gly Val Glu Leu Val Phe Asp
 65 70 75 80
 Gly Lys Arg Val Pro Val His Leu Thr Glu Leu Thr Gly Gly Lys Ser
 85 90 95
 Val Met Val Tyr Gly Gln Thr Glu Val Thr Arg Asp Leu Met Asp Ala
 100 105 110
 Arg Ala Ala Ser Gly Ala Pro Ile Val Tyr Ser Ala Ser Asn Val Glu
 115 120 125
 Leu His Glu Leu Lys Gly Glu Arg Pro Tyr Val Thr Phe Glu Lys Asp
 130 135 140
 Gly Glu Arg Val Arg Val Asp Cys Asp Tyr Ile Ala Gly Cys Asp Gly
 145 150 155 160
 Phe His Gly Val Ser Arg Lys Ser Ile Pro Glu Gly Val Leu Thr Glu
 165 170 175
 Tyr Glu Arg Val Tyr Pro Phe Gly Trp Leu Gly Leu Leu Ser Asp Thr
 180 185 190
 Pro Pro Val His Glu Glu Leu Ile Tyr Ala His His Glu Arg Gly Phe
 195 200 205
 Ser Leu Cys Ser Gln Arg Ser Leu Thr Arg Ser Arg Tyr Tyr Leu Gln
 210 215 220
 Val Pro Leu Thr Asp Lys Val Glu Asp Trp Ser Asp Glu Arg Phe Trp
 225 230 235 240
 Asn Glu Leu Lys Ala Arg Leu Pro Glu Asp Val Ala Ala Lys Leu Val
 245 250 255
 Thr Gly Pro Ser Leu Glu Lys Ser Ile Ala Pro Leu Arg Ser Phe Val
 260 265 270
 Val Glu Pro Met Gln Tyr Gly His Leu Phe Leu Val Gly Asp Ala Ala
 275 280 285
 His Ile Val Pro Pro Thr Gly Ala Lys Gly Leu Asn Leu Ala Ala Ser
 290 295 300
 Asp Val Cys Tyr Leu Tyr Arg Ile Leu Val Lys Val Tyr Arg Glu Gly
 305 310 315 320
 Arg Thr Asp Leu Leu Glu Lys Tyr Ser Glu Leu Ala Leu Arg Arg Val
 325 330 335
 Trp Lys Gly Glu Arg Phe Ser Trp Phe Met Thr Asn Leu Leu His Asp
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 Phe Asp Gly His Lys Asp Ala Trp Asp Gln Lys Met Gln Gln Ala Asp
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 Arg Glu Tyr Phe Leu Gly Ser His Ala Gly Leu Val Asn Ile Ala Glu
 370 375 380

Asn Tyr Val Gly Leu Pro Tyr Glu Asp Val Lys
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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1446 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GATCAGCGCC	GGCGCATGCG	CCGCCGGCCA	GCCATAACAG	CGAGAGGGCT	ATCCGCAATG	60
AAGACTCAAG	TCGCCATCAT	CGGCGCCGGT	CCGTCCGGCC	TCCTGCTCGG	CCAGTTGCTG	120
CACAAGGCCG	GCATCGACAA	CGTGATCCTC	GAACGCCAGA	CCCCGGACTA	CGTGCTCGGC	180
CGCATCCGCG	CCGGCGTGCT	GGAACAGGGT	ATGGTCGACC	TGCTGCGCGA	GGCCGGCGTC	240
GACCGGCGCA	TGGCGCGCGA	CGGGCTGGTC	CACGAAGGCG	TGGAGATCGC	CTTCGCCGGG	300
CAGCGCCGGC	GCATCGACCT	GAAGCGCCTG	AGCGGCGGCA	AGACGGTGAC	GGTCTACGGC	360
CAGACCGAGG	TCACCCGCGA	CCTCATGGAA	GCCCGCGAAG	CCTGCGGCGC	CACTACCGTC	420
TACCAGGCCG	CCGAGGTGCG	CCTGCACGAC	CTGCAAGGTG	AGCGCCCCTA	CGTGACCTTC	480
GAACGCGACG	GCGAACGGCT	ACGCCTGGAT	TGCGACTACA	TCGCCGGCTG	CGATGGCTTC	540
CACGGCATCT	CGCGGCAATC	GATCCCGGCG	GAGCGGCTGA	AGGTCTTCGA	GCGGGTCTAT	600
CCGTTCGGCT	GGCTCGGCCT	GCTCGCCGAC	ACCCCGCCGG	TCAGCCACGA	ACTGATCTAC	660
GCCAACCATC	CGCGCGGCTT	CGCCCTGTGC	AGCCAGCGTT	CGGCGACCCG	CAGCCGCTAC	720
TACGTACAGG	TGCCATTGAC	AGAGAAAGTC	GAGGACTGGT	CCGACGAGCG	CTTCTGGACG	780
GAAGTAAAG	CGCGCCTCCC	GGCCGAGGTG	GCGGAGAAAC	TGGTGACCGG	TCCTTCGCTG	840
GAGAAGAGCA	TCGCGCCGCT	GCGCAGCTTC	GTGGTCGAGC	CGATGCAGCA	TGGCCGGCTG	900
TTCTTCGCCG	GCGACGCCGC	GCACATCGTG	CCGCCACCCG	GCGCCAAGGG	ACTGAACCTG	960
GCGGCCAGCG	ACGTCAGCAC	GCTCTACCGG	CTGCTGCTGA	AGGCCTACCG	CGAAGGGCGG	1020
GGCGAACTGC	TGGAACGCTA	CTCGGCAATC	TGCCTGCGGC	GGATCTGGAA	GGCCGAACGC	1080
TTCTCCTGGT	GGATGACTTC	GGTGCTGCAT	CGCTTCCCCG	ACACCGACGC	GTTCAGCCAG	1140
CGCATCCAGC	AGACCGAACT	GGAGTACTAC	CTGGGCTCCG	AGGCGGGCCT	GGCGACCATC	1200
GCCGAGAACT	ATGTCGGCCT	GCCCTACGAG	GAAATCGAGT	AGGCCGCGCC	ACCCCGGCGC	1260
AACGCCGGGG	CACCGCTCCC	AAGGCGGGAC	GAACGTTGGC	GCAGGCGAAT	ATCGACGAAA	1320
GACGACCCTC	GGACTTCGAT	ATTCTCCGGC	ACCTGTCCAT	CCCCAGGCGC	AGTATGATGC	1380
CCGTGCCTTC	GCGTTCCGTC	CGCCAGGTG	CCGCTCTTGT	CCGATCTGCC	AGAACCCGCC	1440
CGCCGT						1446

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1315 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```

AGGCTTTGTC GCAGTTTCA CCTTCTTATG CTGCCCCTC AAGTGAAAA TGTCAGCCAT   60
AACACCAATG ACAACTCTTT CACTTGATCC GTTCGGGTGA AGCTTGCGAG CCATAAAAAT  120
AATGAAAACG CTA AAAACCC AAGTCGCCAT TATTGGCGCC GGTCCCTCCG GATTGCTGCT  180
CGGCCAGTTA CTGCACAACG CGGGTATCCA GACCCTGCTT CTAGAGCGCC AGAGCGCCGA  240
CTACGTGCAA GGCCGCATCC GTGCCGGGGT GCTGGAGCAA GGCATGGTCG ACCTGCTGCG  300
CGAAGCGGGC GTCAGCCGAC GCATGGACGC CGAGGGCCTT GTGCATGACG GTTTCGAATT  360
GGCACTCAAT GGCGAACTCA CCCACATCGA CCTCAAGGCG CTCACCGGCG GCCAGTCGGT  420
GATGATCTAC GGCCAGACCG AAGTCACCCG TGA CTTGATG GCCGCCCGCG AAGCGGCGGG  480
TGGCATCACT CTATACGAAA CGCAGAACGT GCAGCCTCAT GGTCACAAAA CTGATCGACC  540
CTGGCTGACC TTCGAGCACC AGGGTGAAGC TTTTCGCCTG GAGTGCGACT ACATCGCGGG  600
CTGTGATGGT TTTCACGGTG TGGCGCGGCA GTCGATTCCG GCGCAGTCGT TGAAGGTCTT  660
CGAGCGCGTC TATCCCTTCG GTTGGCTGGG CGTCCTCGCC GACACACCGC CGGTGCATGA  720
CGAACTGGTG TACGCCAAAC ATGCGCGTGG CTTTGCCCTG TGCAGCATGC GCTCGCCGAC  780
CCGCAGCCGC TATTACCTGC AAGTGCCGGT TGAAGAAGCG CTGGATGAAT GGTCCGATCA  840
GCGCTTCTGG GATGAGCTGA AAACCCGTTT GCCCAGTGCA CTGGCGGCCC AACTGGTCAC  900
CGGGCCATCC ATCGAGAAGA GCATCGCGCC CGCTGCGAGC TTTGAGGTCG AGCCGATGCA  960
ATACGGGCGC CTGTTCTCTG TGGGGGACGC CGCGCATATC GTGCCGCCCA CCGGGGCCAA 1020
GGGCTTGAAC CTGGCGGCCA GCGACGTGAG TACGCTGTTT CGGATCTTGC TCAAGGTCTA 1080
TCGCGAGGGG CGGGTGGACC TGCTGGAACA GTACTCAGCG ATCTGCTTGC GCCGCGTATG 1140
GAAAGCCGAA CGGTTTTCTT GGTGGATGAC TTCGATGTTG CAC CAGTTTC CGGAGGCCGA 1200
CGGGTTCAGC CAGCGCATTG CCGAGAGCGA GCTTGCGTAT TTCATCAGCT CCGAGGCGGG 1260
CCGCAA AACC ATCGCAGAAA ATTACGTCGG GCTTCCTTAC GAAGCTATCG AGTAG      1315

```

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 394 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Lys Thr Gln Val Ala Ile Ile Gly Ala Gly Pro Ser Gly Leu Leu
 1 5 10 15
 Leu Gly Gln Leu Leu His Lys Ala Gly Ile Asp Asn Val Ile Leu Glu
 20 25 30
 Arg Gln Thr Pro Asp Tyr Val Leu Gly Arg Ile Arg Ala Gly Val Leu
 35 40 45
 Glu Gln Gly Met Val Asp Leu Leu Arg Glu Ala Gly Val Asp Arg Arg
 50 55 60
 Met Ala Arg Asp Gly Leu Val His Glu Gly Val Glu Ile Ala Phe Ala
 65 70 75 80
 Gly Gln Arg Arg Arg Ile Asp Leu Lys Arg Leu Ser Gly Gly Lys Thr
 85 90 95
 Val Thr Val Tyr Gly Gln Thr Glu Val Thr Arg Asp Leu Met Glu Ala
 100 105 110
 Arg Glu Ala Cys Gly Ala Thr Thr Val Tyr Gln Ala Ala Glu Val Arg
 115 120 125
 Leu His Asp Leu Gln Gly Glu Arg Pro Tyr Val Thr Phe Glu Arg Asp
 130 135 140
 Gly Glu Arg Leu Arg Leu Asp Cys Asp Tyr Ile Ala Gly Cys Asp Gly
 145 150 155 160
 Phe His Gly Ile Ser Arg Gln Ser Ile Pro Ala Glu Arg Leu Lys Val
 165 170 175
 Phe Glu Arg Val Tyr Pro Phe Gly Trp Leu Gly Leu Leu Ala Asp Thr
 180 185 190
 Pro Pro Val Ser His Glu Leu Ile Tyr Ala Asn His Pro Arg Gly Phe
 195 200 205
 Ala Leu Cys Ser Gln Arg Ser Ala Thr Arg Ser Arg Tyr Tyr Val Gln
 210 215 220
 Val Pro Leu Thr Glu Lys Val Glu Asp Trp Ser Asp Glu Arg Phe Trp
 225 230 235 240
 Thr Glu Leu Lys Ala Arg Leu Pro Ala Glu Val Ala Glu Lys Leu Val
 245 250 255
 Thr Gly Pro Ser Leu Glu Lys Ser Ile Ala Pro Leu Arg Ser Phe Val
 260 265 270
 Val Glu Pro Met Gln His Gly Arg Leu Phe Leu Ala Gly Asp Ala Ala
 275 280 285
 His Ile Val Pro Pro Thr Gly Ala Lys Gly Leu Asn Leu Ala Ala Ser
 290 295 300
 Asp Val Ser Thr Leu Tyr Arg Leu Leu Leu Lys Ala Tyr Arg Glu Gly
 305 310 315 320
 Arg Gly Glu Leu Leu Glu Arg Tyr Ser Ala Ile Cys Leu Arg Arg Ile
 325 330 335

Trp Lys Ala Glu Arg Phe Ser Trp Trp Met Thr Ser Val Leu His Arg
 340 345 350
 Phe Pro Asp Thr Asp Ala Phe Ser Gln Arg Ile Gln Gln Thr Glu Leu
 355 360 365
 Glu Tyr Tyr Leu Gly Ser Glu Ala Gly Leu Ala Thr Ile Ala Glu Asn
 370 375 380
 Tyr Val Gly Leu Pro Tyr Glu Glu Ile Glu
 385 390

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 397 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Met Lys Thr Leu Lys Thr Gln Val Ala Ile Ile Gly Ala Gly Pro Ser
 1 5 10 15
 Gly Leu Leu Leu Gly Gln Leu Leu His Asn Ala Gly Ile Gln Thr Leu
 20 25 30
 Leu Leu Glu Arg Gln Ser Ala Asp Tyr Val Gln Gly Arg Ile Arg Ala
 35 40 45
 Gly Val Leu Glu Gln Gly Met Val Asp Leu Leu Arg Glu Ala Gly Val
 50 55 60
 Ser Arg Arg Met Asp Ala Glu Gly Leu Val His Asp Gly Phe Glu Leu
 65 70 75 80
 Ala Leu Asn Gly Glu Leu Thr His Ile Asp Leu Lys Ala Leu Thr Gly
 85 90 95
 Gly Gln Ser Val Met Ile Tyr Gly Gln Thr Glu Val Thr Arg Asp Leu
 100 105 110
 Met Ala Ala Arg Glu Ala Ala Gly Gly Ile Thr Leu Tyr Glu Thr Gln
 115 120 125
 Asn Val Gln Pro His Gly His Lys Thr Asp Arg Pro Trp Leu Thr Phe
 130 135 140
 Glu His Gln Gly Glu Ala Phe Arg Leu Glu Cys Asp Tyr Ile Ala Gly
 145 150 155 160
 Cys Asp Gly Phe His Gly Val Ala Arg Gln Ser Ile Pro Ala Gln Ser
 165 170 175
 Leu Lys Val Phe Glu Arg Val Tyr Pro Phe Gly Trp Leu Gly Val Leu
 180 185 190
 Ala Asp Thr Pro Pro Val His Asp Glu Leu Val Tyr Ala Lys His Ala
 195 200 205
 Arg Gly Phe Ala Leu Cys Ser Met Arg Ser Pro Thr Arg Ser Arg Tyr
 210 215 220

Tyr Leu Gln Val Pro Val Glu Glu Ala Leu Asp Glu Trp Ser Asp Gln
 225 230 235 240
 Arg Phe Trp Asp Glu Leu Lys Thr Arg Leu Pro Ser Ala Leu Ala Ala
 245 250 255
 Gln Leu Val Thr Gly Pro Ser Ile Glu Lys Ser Ile Ala Pro Ala Ala
 260 265 270
 Ser Phe Glu Val Glu Pro Met Gln Tyr Gly Arg Leu Phe Leu Leu Gly
 275 280 285
 Asp Ala Ala His Ile Val Pro Pro Thr Gly Ala Lys Gly Leu Asn Leu
 290 295 300
 Ala Ala Ser Asp Val Ser Thr Leu Phe Arg Ile Leu Leu Lys Val Tyr
 305 310 315 320
 Arg Glu Gly Arg Val Asp Leu Leu Glu Gln Tyr Ser Ala Ile Cys Leu
 325 330 335
 Arg Arg Val Trp Lys Ala Glu Arg Phe Ser Trp Trp Met Thr Ser Met
 340 345 350
 Leu His Gln Phe Pro Glu Ala Asp Gly Phe Ser Gln Arg Ile Ala Glu
 355 360 365
 Ser Glu Leu Ala Tyr Phe Ile Ser Ser Glu Ala Gly Arg Lys Thr Ile
 370 375 380
 Ala Glu Asn Tyr Val Gly Leu Pro Tyr Glu Ala Ile Glu
 385 390 395

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
- (A) DESCRIPTION: /desc = "PRIMER"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CARTTRCTNC AYAANGTNGG NAT

23

Applicant's or agent's file reference number	CR9967A	International application No.	Unknown
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>3</u> , line s <u>29 - 32</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (including postal code and country) 10801 University Blvd. Manassas, Virginia 20110-2209 USA	
Date of deposit 26 November 1996	Accession Number 55885
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

<p>For receiving Office use only</p> <p><input checked="" type="checkbox"/> This sheet was received with the international application</p> <p>Authorized officer Sonya Barnes PCT International Division</p>	<p>For International Bureau use only</p> <p><input type="checkbox"/> This sheet was received by the International Bureau on:</p> <p>Authorized officer</p>
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Applicant's or agent's file reference number	Ck9967A	International application No.	Unknown
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>3</u> , line s <u>33 - 36</u>	
B. IDENTIFICATION OF DEPOSIT	
Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (including postal code and country) 10801 University Blvd. Manassas, Virginia 20110-2209 USA	
Date of deposit 26 November 1996	Accession Number 55886
C. ADDITIONAL INDICATIONS (leave blank if not applicable)	
This information is continued on an additional sheet <input type="checkbox"/>	
In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g. "Accession Number of Deposit")	

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<input checked="" type="checkbox"/> This sheet was received with the international application	<input type="checkbox"/> This sheet was received by the International Bureau on:
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WHAT IS CLAIMED IS:

1. A method for the production of PHBA comprising:
 - (i) culturing a *pobA(-)* *Pseudomonas* strain in a medium containing an aromatic organic substrate, at least one suitable carbon source, and a nitrogen source, wherein the *pobA(-)* *Pseudomonas* strain comprises *pobA* genes encoding the TMO toluene degradation pathway and wherein the *pobA(-)* *Pseudomonas* strain does not produce any detectable *para*-hydroxybenzoate hydroxylase, whereby PHBA accumulates; and
 - (ii) recovering the PHBA.
2. The method of Claim 1 wherein the genes encoding the TMO toluene degradation pathway of the *pobA(-)* *Pseudomonas* strain are characterized by a disruption in at least two *pobA* genes.
3. The method of Claim 1 wherein the *pobA(-)* *Pseudomonas* strain is selected from the group consisting of KRC1651 (ATCC 55885), KRC1616, and KRC1615.
4. The method of Claim 2 wherein at least one *pobA* gene encodes a *para*-hydroxybenzoate hydroxylase enzyme having a first amino acid sequence as set out in SEQ ID NO:3 or a second amino acid sequence as set out in SEQ ID NO:4 and wherein the first and second amino acid sequence may encompass amino acid substitutions, deletions or additions that do not alter the function of the *para*-hydroxybenzoate hydroxylase.
5. The method of Claim 2 wherein at least one *pobA* gene is *pobA-1* and is defined by SEQ ID NO:1.
6. The method of Claim 2 wherein at least one *pobA* gene is *pobA-2* and is defined by SEQ ID NO:2.
7. The method of Claim 1 wherein the aromatic organic substrate is selected from the group consisting of toluene, *p*-cresol, *p*-hydroxybenzyl alcohol, benzoate and *p*-hydroxybenzaldehyde.
8. The method of Claim 7 wherein toluene is present in the medium in a concentration of less than 500 ppm.
9. The method of Claim 7 wherein toluene is present in the medium from about 30 ppm to about 60 ppm.
10. The method of Claim 1 wherein the suitable carbon source is succinate, lactate, acetate, ethanol, monosaccharides, oligosaccharides, polysaccharides, or mixtures thereof.
11. The method of Claim 10 wherein the suitable carbon source is glucose or succinate.
12. The method of Claim 1 wherein PHBA accumulates at a concentration of at least 1 mM.

13. The method of Claim 1 wherein PHBA accumulates at a rate of about 0.01 g PHBA/g-cells-h to about 1 g PHBA/g-cells-h.
14. The method of Claim 1 wherein the *pobA*(-) *Pseudomonas* strain is cultured at a pH of from about 6.3 to about 7.9.
- 5 15. The method of Claim 1, the medium further containing a Mg ion at a concentration of about 2 mM to about 12 mM.
16. A *pobA* gene encoding a *para*-hydroxybenzoate hydroxylase enzyme having the amino acid sequence as shown by SEQ ID NO:3 or SEQ ID NO:4.
17. The gene of Claim 16 wherein the gene is *pobA-1* and is defined by
10 SEQ ID NO:1 or is *pobA-2* and is defined by SEQ ID NO:2.
18. A transformed *pobA*(-) *Pseudomonas* strain comprising an amino acid disruption in at least two *pobA* genes.
19. The transformed *pobA*(-) *Pseudomonas* strain of Claim 18 selected
15 from the group consisting of KRC1651 (ATCC 55885), KRC1616, and KRC1615.
20. A *Pseudomonas* strain selected from the group consisting of biological deposits identified as KRC1651 (ATCC 55885) and KRC16 (ATCC 55886).
21. A TMO *P. mendocina* variant having a cell density at least 6 fold greater than the wildtype under growth conditions of approximately 160ppm
20 toluene, the TMO *P. mendocina* variant produced by mutagenesis of the wildtype KR-1.
22. The TMO *P. mendocina* variant of Claim 21 selected from the group consisting of KRC16 and KRC50.

FIG. 1a

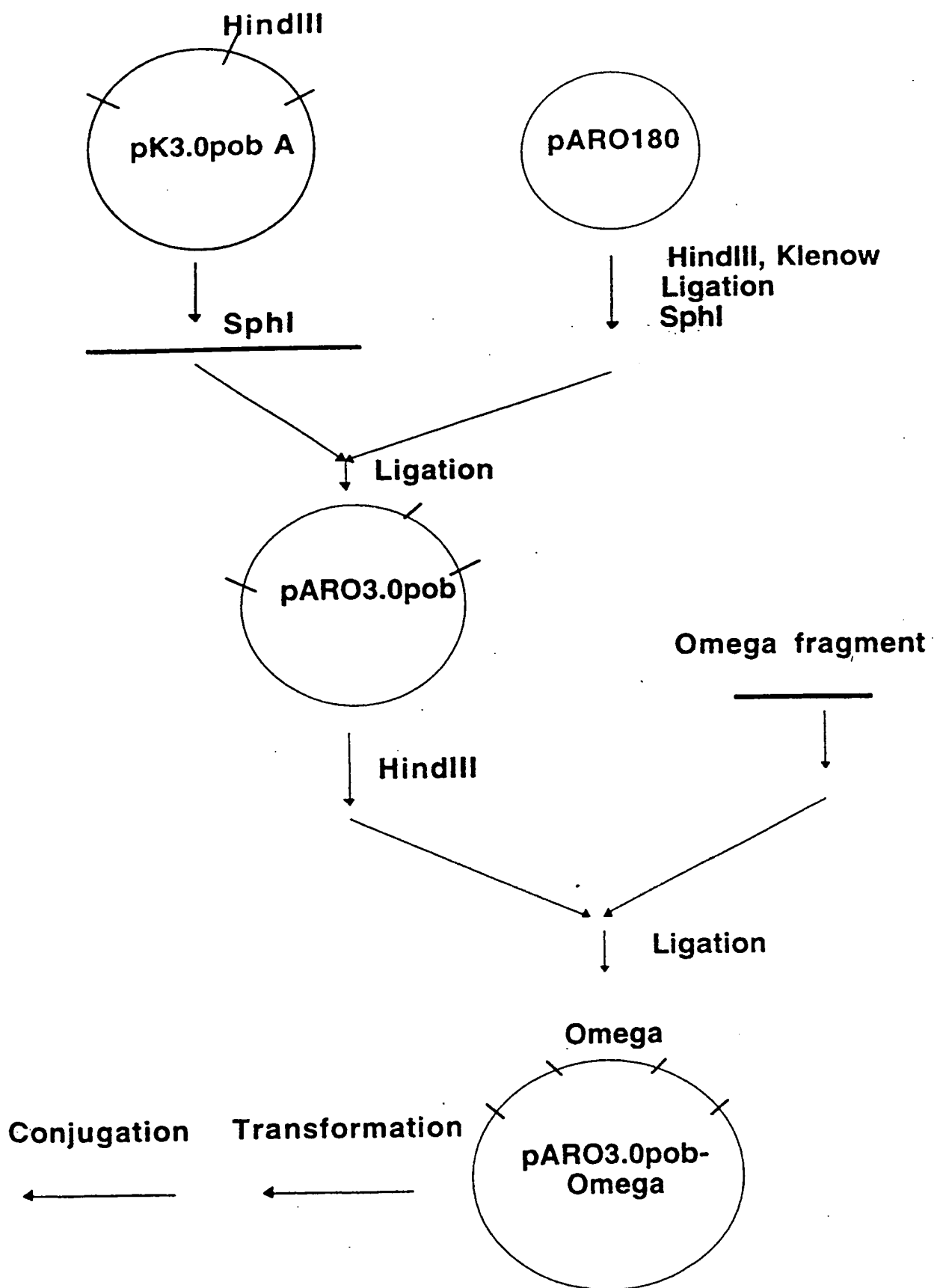


FIG.1b

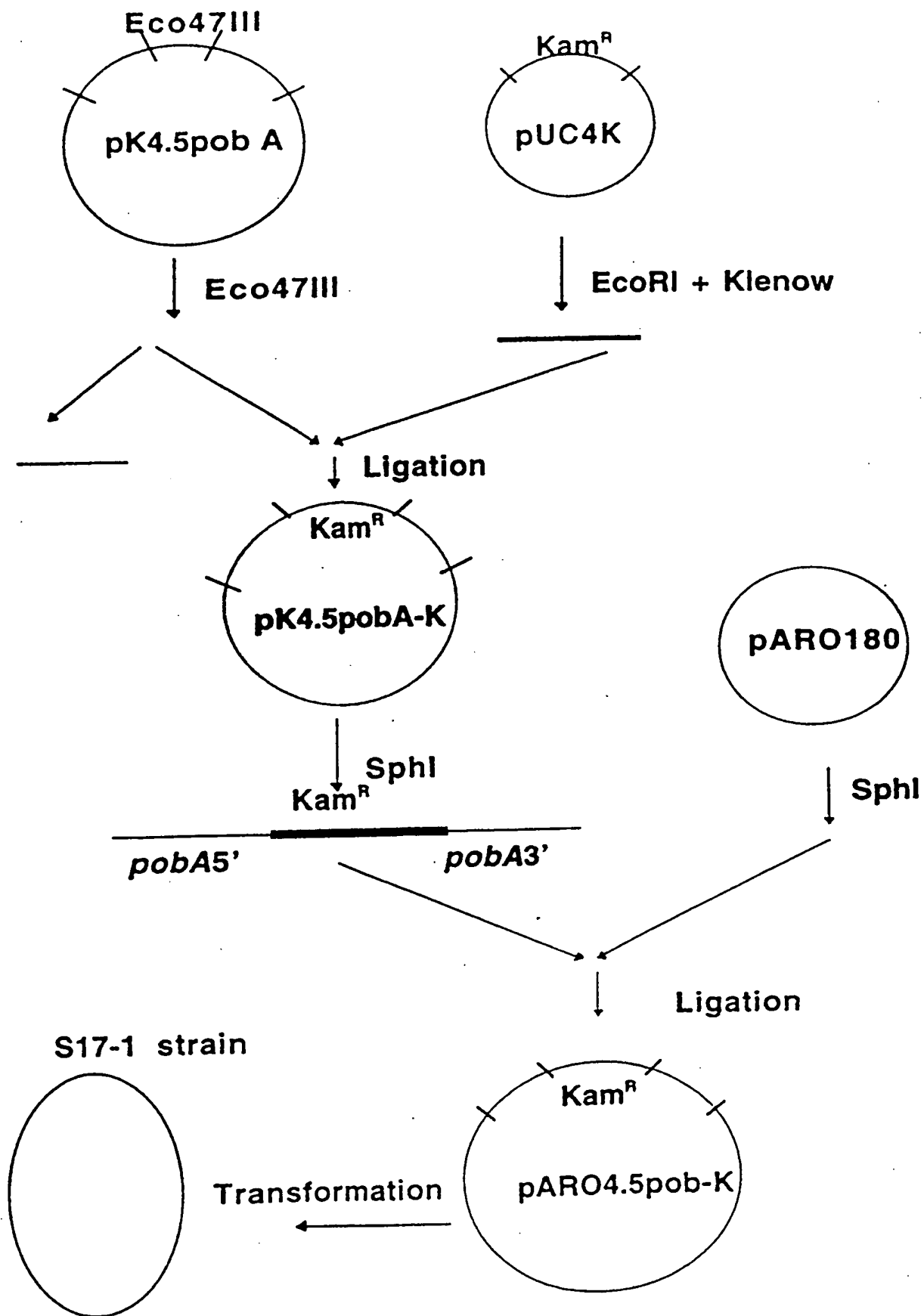


FIG.2

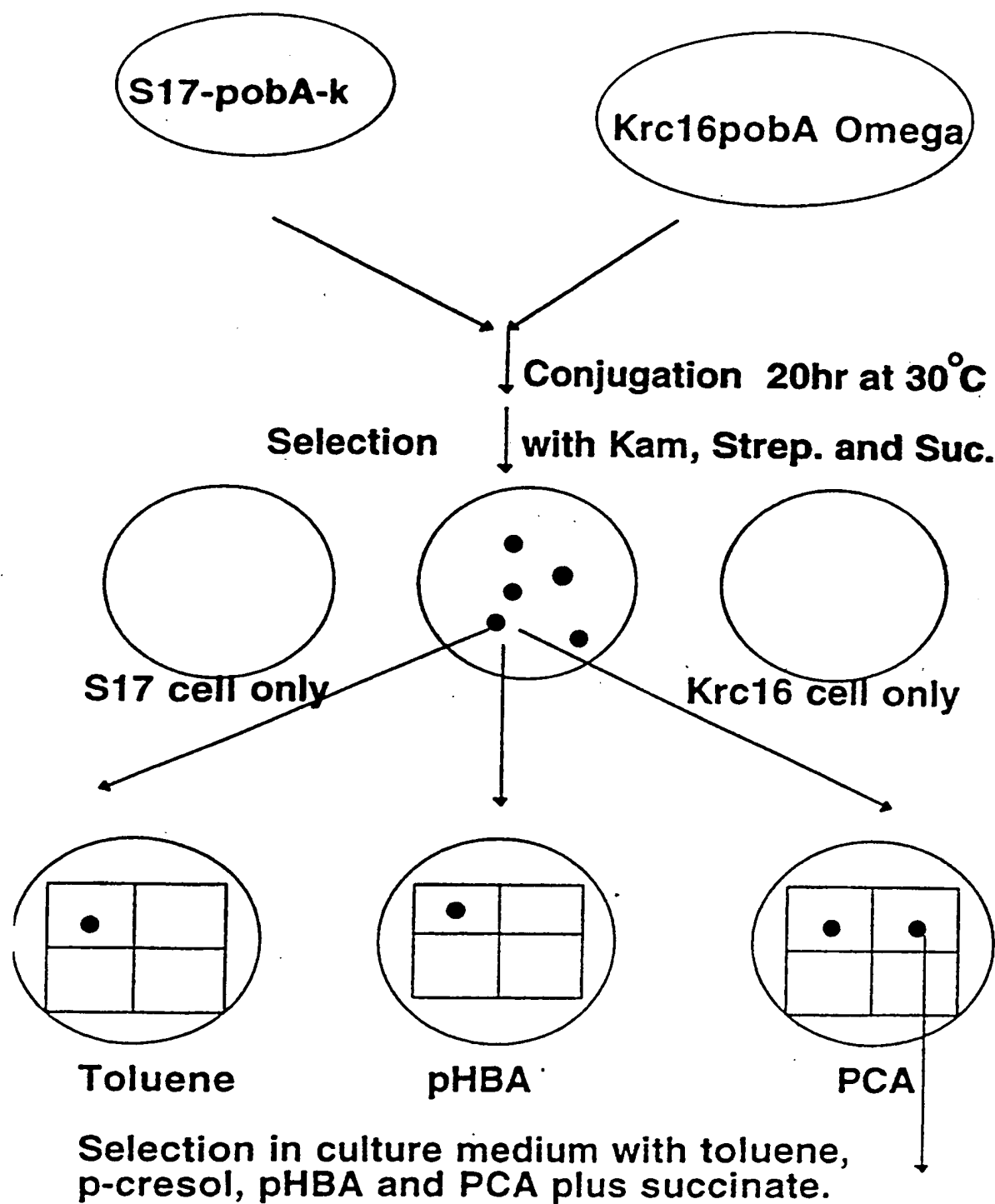


FIG. 3

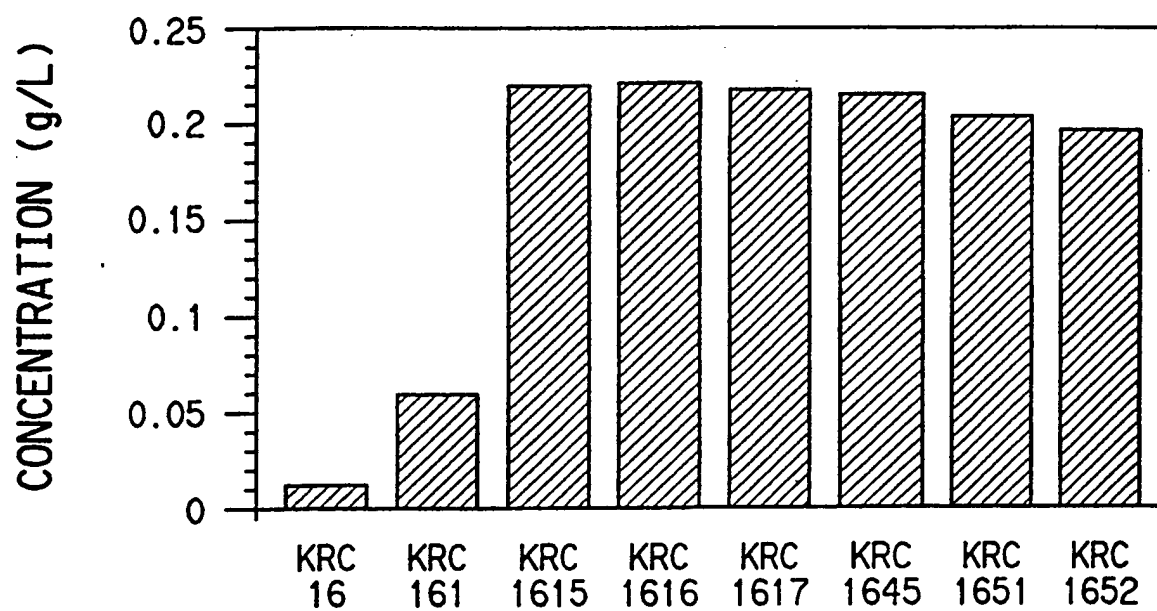


FIG. 4

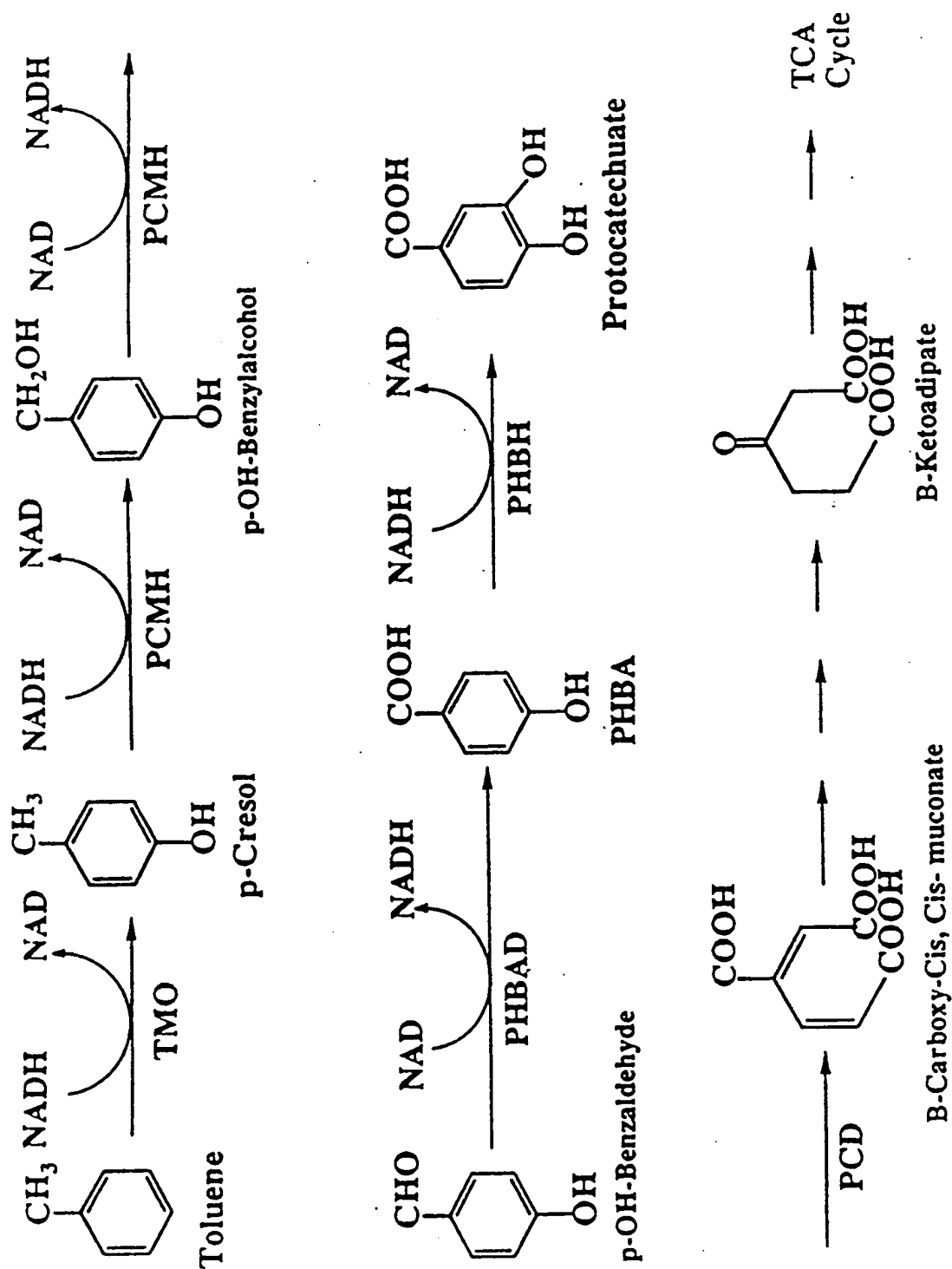
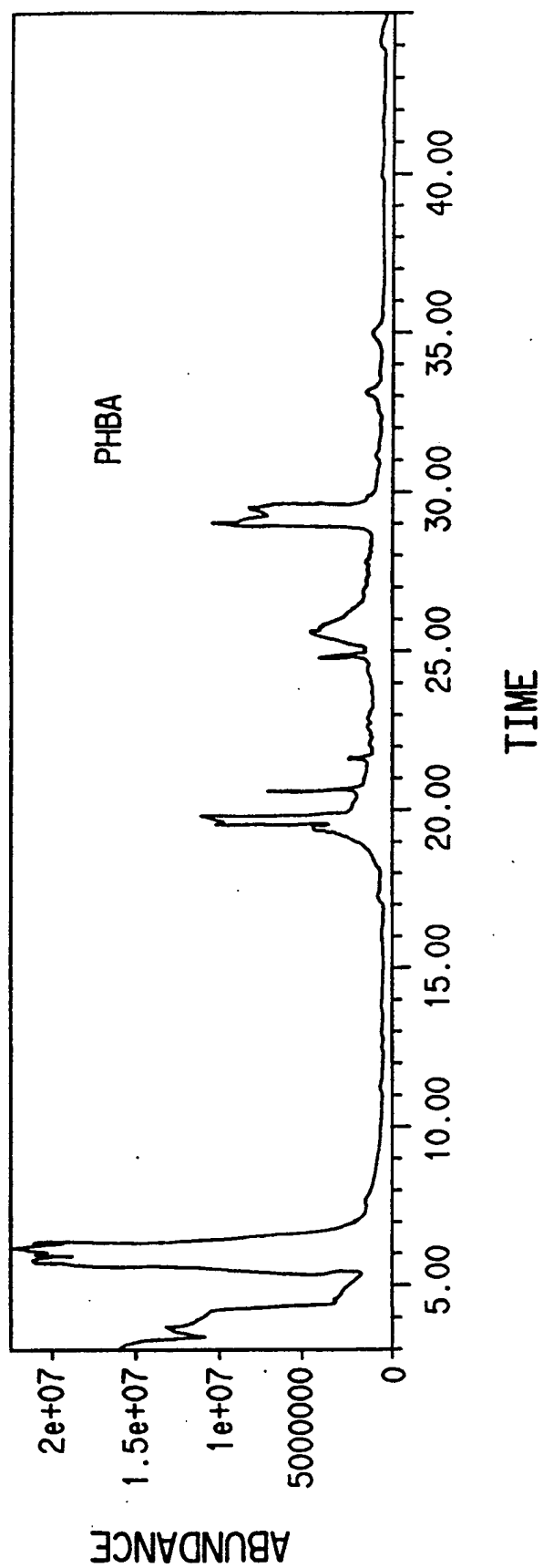


FIG. 5



INTERNATIONAL SEARCH REPORT

Int. Application No

PCT/US 98/12072

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/52 C12N15/53 C12N9/02 C12P7/42 C12N1/21
 //(C12N1/21,C12R1:38)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 122, no. 5, 30 January 1995 Columbus, Ohio, US; abstract no. 48056, WONG, CHERYL M. ET AL: "Cloning and sequencing show that 4-hydroxybenzoate hydroxylase (PcbA) is required for uptake of 4-hydroxybenzoate in Rhizobium leguminosarum" XP002080559 see abstract & MICROBIOLOGY (READING, U. K.) (1994), 140(10), 2775-86 CODEN: MROBEO;ISSN: 1350-0872, ----- -/--	1



Further documents are listed in the continuation of box C.



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* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

13 October 1998

Date of mailing of the international search report

23/10/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
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 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
 Fax: (+31-70) 340-3016

Authorized officer

Delanghe, L

INTERNATIONAL SEARCH REPORT

Int'l Application No

PCT/US 98/12072

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>ENTSCH, BARRIE ET AL: "Sequence and organization of pobA, the gene coding for p-hydroxybenzoate hydroxylase, an inducible enzyme from Pseudomonas aeruginosa"</p> <p>GENE (1988), 71(2), 279-91 CODEN: GENED6; ISSN: 0378-1119, XP002080557</p> <p>cited in the application</p> <p>see the whole document</p> <p style="text-align: center;">---</p>	1
A	<p>BARRY SHUMAN ET AL.: "Cloning, nucleotide sequence, and expression of a p-hydroxybenzoate hydroxylase isozyme gene from Pseudomonas fluorescens."</p> <p>JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 268, no. 23, 15 August 1993, page 17057-17062 XP002080558</p> <p>MD US</p> <p>cited in the application</p> <p>see the whole document</p> <p style="text-align: center;">---</p>	1
A	<p>WO 92 06208 A (AMGEN INC) 16 April 1992</p> <p>see claims</p> <p style="text-align: center;">-----</p>	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/12072

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9206208 A	16-04-1992	US 5171684 A	15-12-1992
		AU 651142 B	14-07-1994
		AU 8651391 A	28-04-1992
		EP 0504349 A	23-09-1992
		JP 5502593 T	13-05-1993
		US 5605823 A	25-02-1997
<hr/>			

FIG. 3

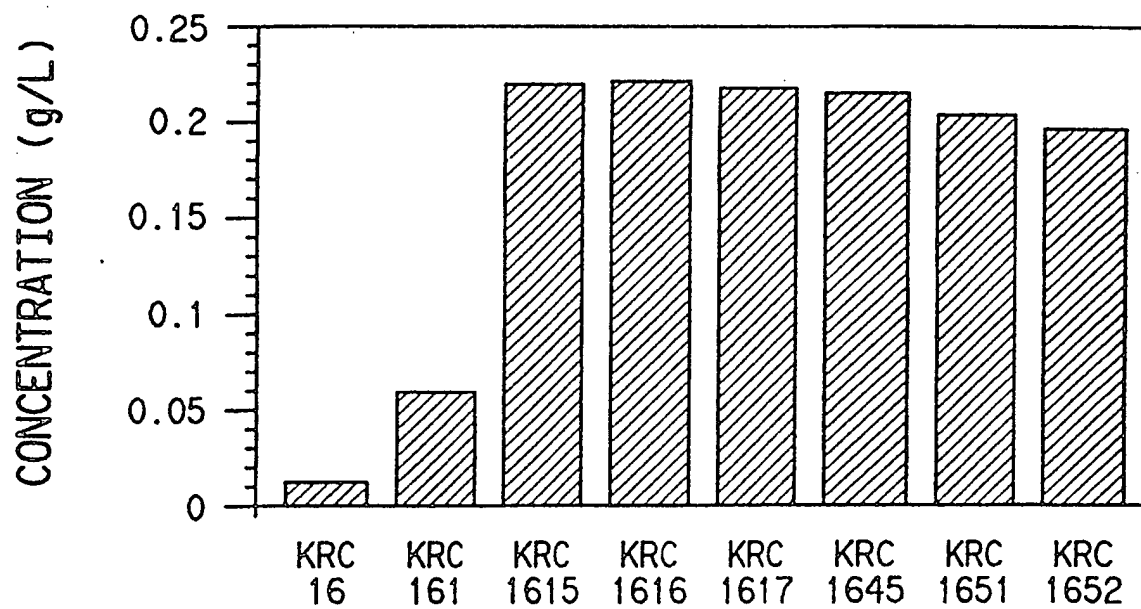


FIG. 4

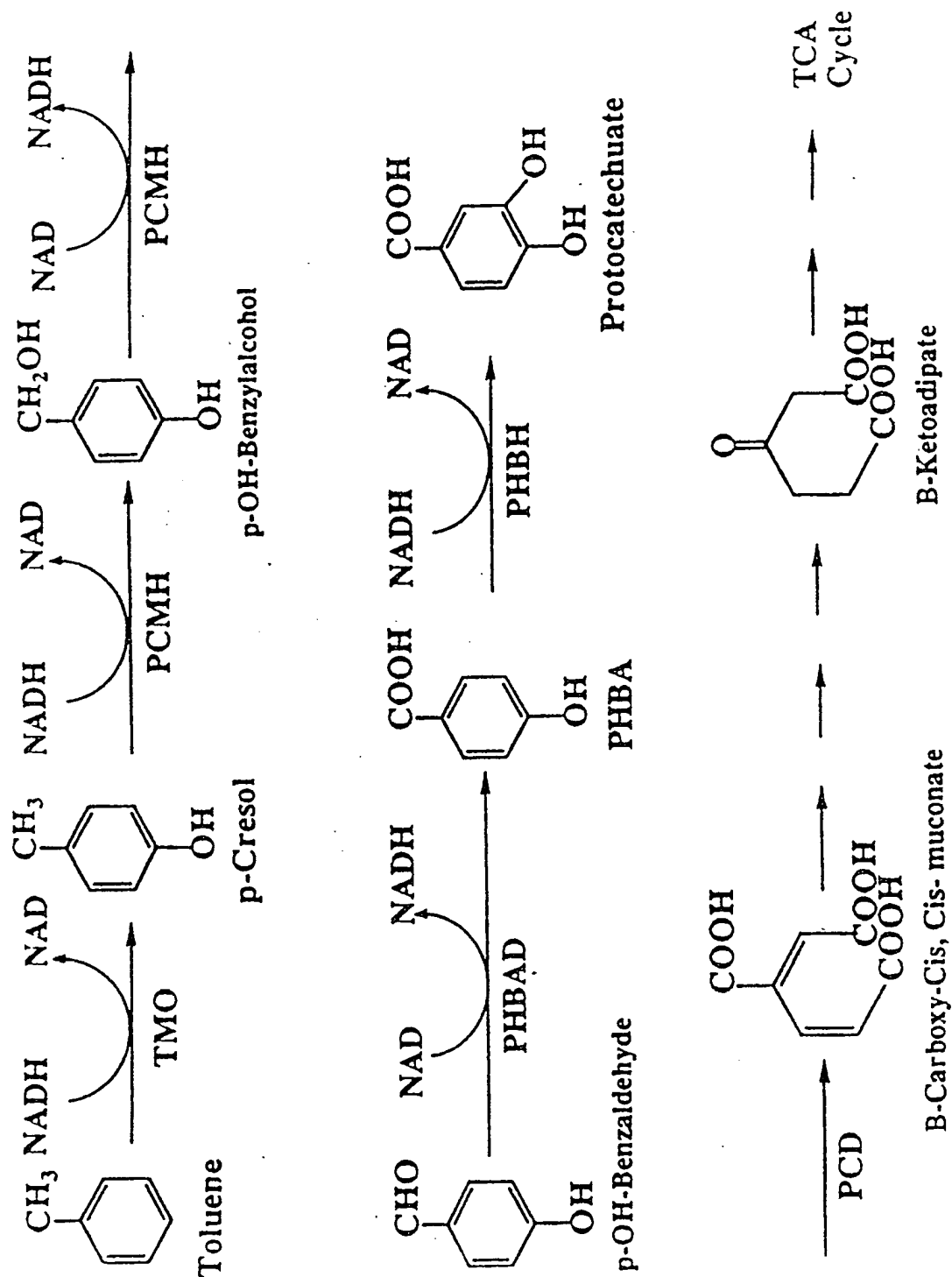
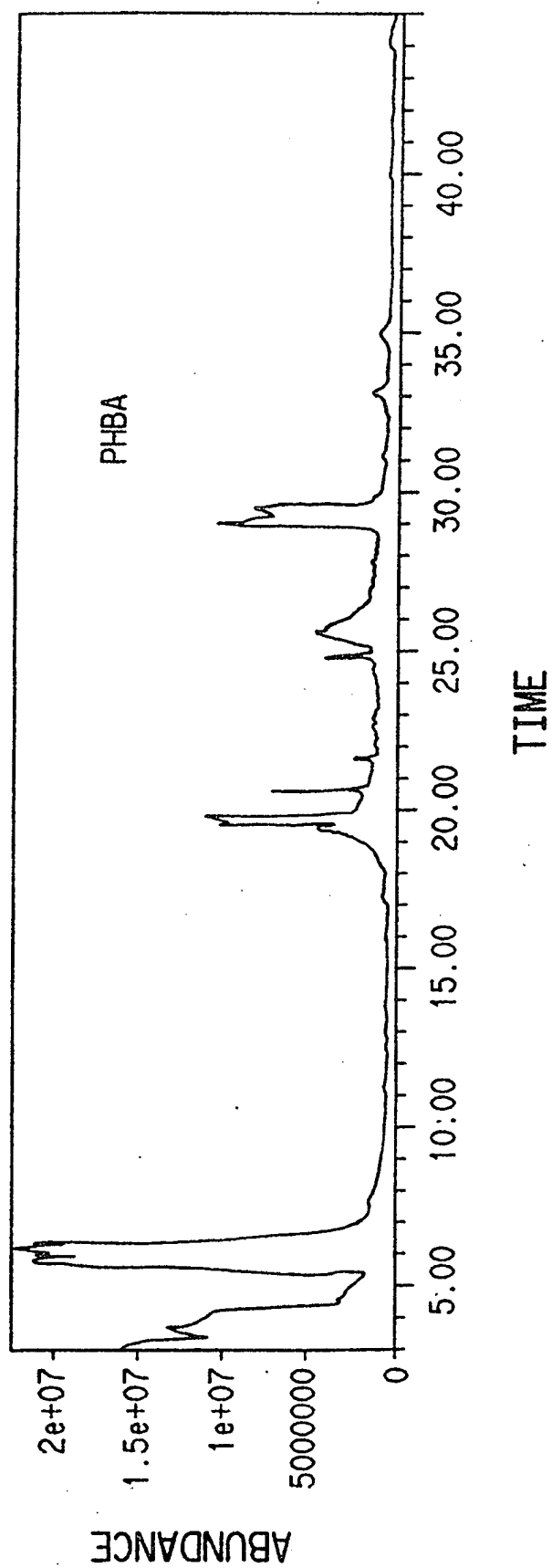


FIG. 5



INTERNATIONAL SEARCH REPORT

Int. l. Application No
PCT/US 98/12072

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/52 C12N15/53 C12N9/02 C12P7/42 C12N1/21
//(C12N1/21,C12R1:38)

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 122, no. 5, 30 January 1995 Columbus, Ohio, US; abstract no. 48056, WONG, CHERYL M. ET AL: "Cloning and sequencing show that 4-hydroxybenzoate hydroxylase (PcbA) is required for uptake of 4-hydroxybenzoate in Rhizobium leguminosarum" XP002080559 see abstract & MICROBIOLOGY (READING, U. K.) (1994), 140(10), 2775-86 CODEN: MROBEO; ISSN: 1350-0872, ----- -/--	1

☒ Further documents are listed in the continuation of box C.

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"O" document referring to an oral disclosure, use, exhibition or other means

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